Sex pheromone communication in the turnip moth *Agrotis segetum*

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DISSERTATION
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PREFACE

When I began my university studies in 1975 the unnatural division of ecology into plant and animal ecology was a popular topic for discussions among students in biology. It seemed to be the outcome of tradition rather than a rational organisation of science. After a few years of courses in biology and chemistry, I started to work on my doctoral thesis in insect pheromone communication. I became affiliated with the Department of Animal Ecology, even though many traditional ecologists might have preferred to label my research chemical ecology. But

> "What's in a name? That which we call a rose
> By any other name would smell as sweet."
>
> (Juliet to Romeo, in Shakespear's play about Romeo and Juliet).

Chemically mediated interactions between organisms are pervasive and important in structuring natural communities. In my opinion, the only possible reason to sort the study of these interactions out from ecology in general (plant or animal?!) would be the different methodology employed. Admittedly a gas chromatograph is very different from a pair of binoculars or telemetric equipment, but never the less it is a very useful ecological tool - especially if combined with a mass spectrometer. The knowledge of pheromone communication has developed tremendously since the late 1950's, when the first pheromone - the silkworm sex attractant - was characterized. Undoubtedly a major reason for this is the invention of gas chromatography and its development to a high level of sophistication. Whereas the identification of the first pheromone required extracts from hundreds of thousands of moths, it is possible today to analyse the pheromone composition of individual female moths and answer pertinent ecological questions on sexual selection and niche separation, as well as on the inheritance of chemical languages and the evolution of chemical signalling.

Looking back I have very much enjoyed working on the sex pheromone communication in the turnip moth - an important pest on carrots and beet roots, especially in southern Europe. A major reason for my pleasant time is the outstanding working facilities and the nice every-day-contacts with

friends and colleagues in the Pheromone group, Ecology Building, Lund University. First of all I would like to express my sincere thanks to Jan Löfqvist, my supervisor and the coordinator of the research project "Odour Signals for Control of Pest Insects", within which my work is integrated. The support that Jan has given me goes far beyond what is obvious from the papers presented in this thesis. It was also Jan who suggested to me that I should work on the fascinating problem of variation in species specific communication systems. The potential use of any new knowledge in future integrated pest control programmes has not made the work less interesting from a scientific point of view - rather the reverse! I am also thankful to professor Per Brinck, head of the Department of Animal Ecology, for his kind provision of facilities and support for the project.

I shared my office with Fredrik Schlyter and his spontaneous comments on ideas and "zero draft" manuscripts have been very stimulating. The help of Ingrid Cederholm, Erling Jirle, Jörgen Jönsson, Elisabeth Marling, Kill Persson, and Elisabeth Wijk has been skilful and indispensable. Jan Van Der Pers was instrumental in teaching me a lot about electrophysiology during three years of cooperation. Charlie Linn taught me how to get moths to become cooperative in a flight tunnel during his short stay in our lab. To both of them I owe many thanks for the know-how they communicated and for their friendship. To those fighting mold and cannibalism to maintain the turnip moth cultures and to all other members of the Pheromone group: Thanks a lot!

With an ambition to walk on two legs, ecology and chemistry, I spent a lot of time in the Laboratory of Ecological Chemistry. I am grateful to Göran Odham, head of the laboratory, for his readiness to help as soon as I encountered chemical difficulties and to Gunilla Westerdahl who tried to teach me how to run the mass spectrometer. Peter Sundin gave up half of his lab-bench for my benefit. There is no way to thank him in a department where space is a limited resource..... When every experiment went wrong and heaven was about to come down, beer-drinking with Anders Tunlid and his friendship promoted my rejuvenation. To those and all the others around the lunch-table: Thanks a lot!

My work has also involved frequent contacts with colleagues outside the own laboratories. I very much enjoyed the hospitality of my friends at the Department of Ecological Chemistry in Göteborg and especially the fruitful cooperation of Monica Appelgren, Gunnar Bergström, and Boel Lanne. The contacts with collaborators at the Department of Organic Chemistry 3 in Lund were highly appreciated: Marie Bengtsson and Bernt Thelin offered altruistic synthesis of pheromone components and Tommy Liljefors has been a continuous

source of valuable suggestions and encouragement. Per Douwes at the Department of Zoology, Lund, kindly helped with the identification of moths. I enjoyed discussing evolutionary aspects of pheromone communication with Sigfrid Lundberg in our own Department and with Bengt-Olle Bengtsson at the Department of Genetics, Lund.

Finally, I am indebted to those who offered me professional help in preparing the manuscripts: John Byers and Marvin Piwoni not only revised my English but kindly provided helpful reviews, Anne Olsson and Anne Odham typed numerous versions of the manuscripts, Steffi Douwes and Astrid Ulfstrand made nice figures out of my lousy sketches, Erling (again - publicity hound!) and Gunilla Lindqvist made it all come together in a nice-looking booklet with an artistic cover by Martin Kornhall. Without their help my work would have been far more laborious, not to mention of poorer quality.

ABSTRACT

The pheromone gland of the female turnip moth Agrotis segetum was found by gas chromatographic analysis to contain at least 18 acetates and alcohols of possible relevance to pheromone communication. Analysis of fatty acids that are possible pheromone precursors in the gland indicate that most of the volatile gland constituents can be derived from the interaction of a delta-11 desaturase and a chain-shortening enzyme with the products of normal fatty acid metabolism.

The role of the behaviourally active homologous acetates (Z)-5-decenyl acetate Z5-10:OAc, Z7-12:OAc and Z9-14:OAc was investigated in field experiments and in a flight tunnel. None of the compounds could be assigned a specific function, but all of them affected all steps in the attraction of males. Each of the compounds is received by specialized receptor cells on the male antenna, but the frequency of the Z9-14:OAc receptor is only 2 %.

The use of gas chromatography- mass spectrometry in the selected ion monitoring mode provided sensitive means of quantifying the pheromone of individuals. A laboratory culture of moths did not differ from a newly collected wild strain, but both populations demonstrated a high variation in the pheromone composition. In field experiments this variation corresponded to a twofold difference in the attraction of males at the most. The existence of pheromone dialects in European populations of the turnip moth was revealed by analysis of moths from Sweden, France, England and Hungary. An aberrant male response of French males to pure Z5-10:OAc, reported by others, was found to correlate with a higher frequency of Z5-10:OAc receptors on the male antenna and a higher relative titre of Z5-10:OAc in French females. Theoretically there should be low additive genetic variance in characters closely related to fitness (such as sexual attractants). The evolutionary basis for the individual variation in pheromone composition and the pheromone dialects is discussed.

LIST OF PAPERS

I Löfstedt, C., Van Der Pers, J.N.C., Löfqvist, J., Lanne, B.S., Appelgren, M., Bergström, G., and Thelin, B. 1982. Sex pheromone components of the turnip moth, Agrotis segetum: Chemical identification, electrophysiological evaluation and behavioural activity. J. Chem. Ecol. 10:1305-1321.

II Van Der Pers, J.N.C. and Löfstedt, C. 1983. Continuous single sensillum recording as a detection method for moth pheromone components in the effluent of a gas chromatograph. Physiol. Entomol: 8:203-211.

III Löfstedt, C., Linn, C.E. Jr., and Löfqvist, J. 1984. Behavioural response of turnip moth males Agrotis segetum to sex pheromone in a flight tunnel and in the field. Manuscript submitted to J. Chem. Ecol.

IV Löfstedt, C., and Odham, G. 1984. Analysis of moth pheromone acetates by selected ion monitoring using electron impact and chemical ionization mass spectrometry. Biomedical Mass Spectrometry 11:106-113.

V Löfstedt, C., Lanne, B.S., Löfqvist, J., Appelgren, M., and Bergström, G. 1983. Individual variation in the pheromone of the turnip moth Agrotis segetum. Manuscript submitted to J. Chem. Ecol.

VI Löfstedt, C., Löfqvist, J., Lanne, B.S., Van Der Pers, J.N.C., and Hansson, B.S. 1984. Pheromone dialects in European turnip moths Agrotis segetum. Manuscript.

INTRODUCTION AND SUMMARY OF RESULTS

THE PROBLEM OF FINDING A MATE

To find a mate is central to every sexually reproducing organism. In many insects the sexual communication and mate seeking behaviour take place by means of chemical signals. In moths, individuals of one sex attract potential mates of the other sex by sexual pheromones. In the vast majority of moth species the female is the calling sex, while males are the responders. Everything else being equal, the selection should be stronger on males than on females to respond over a distance to cues indicating the presence of a member of the opposite sex ready to mate. In most populations the number of females available for mating is limited compared to the number males can fertilize. This results in competition for mates among males. In this respect, moths are representative of most insects in which pair formation is mediated by communication over a distance.

Generally competition is regarded as a major force moulding the structure of natural communities. This has influenced the analysis of moth pheromone communication (for a review, see Cardé and Baker 1984). Insects communicating with chemical signals tend to partition the sex communication channel to ensure the recognition of conspecific members of the opposite sex and to prevent hybridization. (Fig. 1). This might be achieved by species specific pheromone components. Other means to maintain reproductive isolation are the use of the same pheromone components, but in different ratios or the restriction of mating activities to species specific times of the day. I would call this type of conceptual interpretation of moth pheromone communication an adaptationist approach. The demonstration of its validity is as difficult as the demonstration of competition as a selective force in other areas of ecology. In many cases when experimental support is missing, random evolutionary processes could be an alternative explanation for what has been observed.

CHEMICAL AND BIOLOGICAL ANALYSIS OF MOTH PHEROMONES

Moth sexual attractants have been intensively investigated for more than 20 years, following the first characterization of such a compound - the silkworm sex attractant - by Butenandt and coworkers in 1959. However, qualitative and quantitative analyses of moth pheromones still constitute delicate chemical problems. The moth sex pheromones are generally produced in nanogram

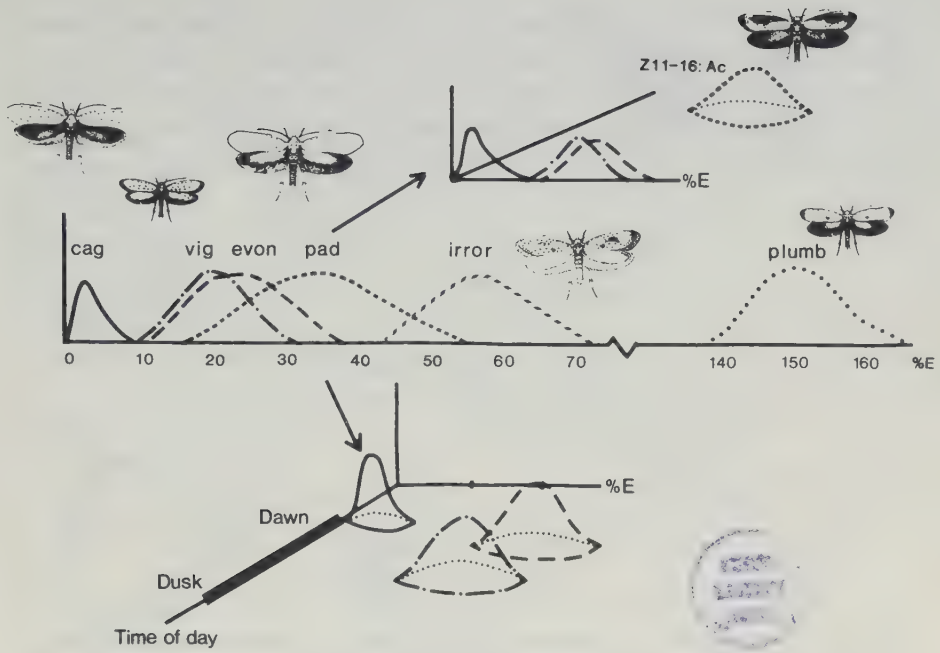

Figure 1. Graphical model of niche separation between small ermine moths, Yponomeuta in the pheromone communication channel. The pheromones of the small ermine moths contain a mixture of two acetates (Z11 and E11-14:OAc). Although, the ratios differ they are not species specific; the niches of some species overlap along the Z/E-axis. One of the species has a third pheromone component (Z11-16:OAc) providing species specificity. Two of the species with overlapping Z/E-ratios are differentiated in a temporal niche dimension; they communicate and mate at different times of day. Abbreviations: cag=cagnagellus, vig=vigintipunctatus, evon=evonymellus, pad=padellus, irror=irrorellus, plumb=plumbellus. (Data mainly from Löfstedt and Van Der Pers, in press and Löfstedt and Herrebout unpublished.)

(10^{-9}g) or subnanogram quantities per individual (Fig. 2). Pheromone compounds in these minute quantities can be chemically characterized by the use of gas chromatography and gas chromatography coupled with mass spectrometry.

Usually insect pheromones are isolated by one of two techniques: by preparation of extracts from pheromone glands or by collection of the odours given off by sexually active insects. Such samples are often very complex, but can be resolved by gas chromatography. Different compounds will pass through the gas chromatographic column at different speeds, according to their chemical properties. If the gas chromatograph is linked with a mass spectrometer, a fingerprint of the molecule structure can be obtained immediately after the separation.

The identification of all compounds in an insect-derived sample is counterproductive because many compounds are not biologically relevant. The

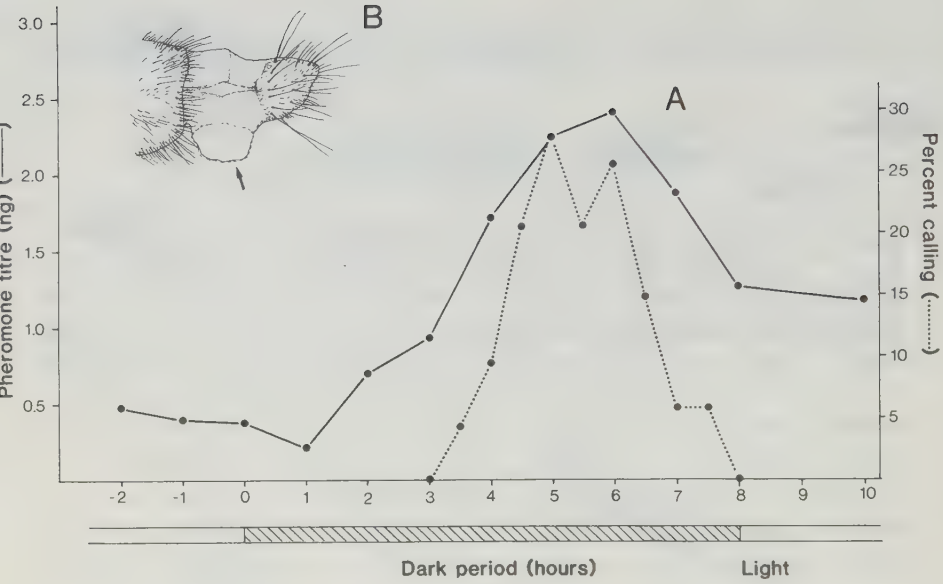

Figure 2. Calling activity (emission of pheromone) and pheromone titre in female turnip moths (A) (Löfstedt & Löfqvist unpublished) and a schematic drawing of the ovipositor with the gland (B) (from Otto et al. 1976). Calling is restricted to certain hours of the day. The gland titre of the pheromone also has a diel periodicity.

cooperation of the insect under study is usually required to designate compounds of behavioural relevance for further chemical characterization. In male moths the antenna is equipped with thousands of sensilla trichodea, innervated with receptor cells that are specialized detectors for female pheromone components. If the antenna is mounted between two electrodes at the outlet of a gas chromatograph, the change in receptor potentials resulting from stimulation can be monitored. Only pheromone components will activate this biological detector, while a conventional chemical detector will register most compounds (Fig. 3). When the reaction of the whole antenna is registered this is called an electroantennogram (EAG). In this case the summarized response of all receptor cells is recorded. If contact is made with a single sensillum, the response of individual receptor cells can be monitored. In this way the specific cells involved in pheromone reception can be characterized.

The assessment of behavioural activity is the ultimate goal of pheromone analysis. Pheromone released behaviour in moths can be studied under controlled and reproducible conditions in the laboratory. A specially designed flight tunnel (Fig. 4A) allows the characterisation of male behaviours in response to females, to odours isolated from females or to synthetic pheromone

blends. A male moth activated by a potent female stimulus will typically react by taking flight, orienting flight to find the odour plume, upwind flight and finally landing and copulation efforts directed towards the female - or the synthetic lure. With access to a continuous laboratory culture of suitable insects, pheromone behaviour can be studied all-year-round in this way. Field

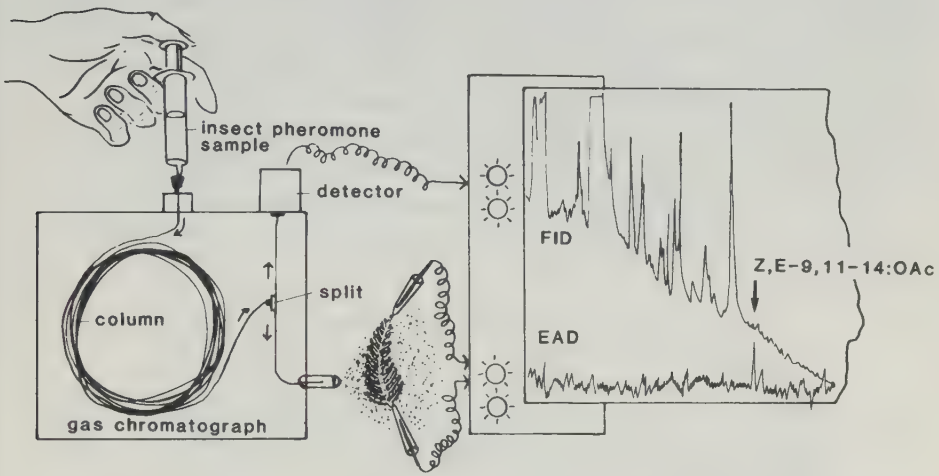

Figure 3. Simultaneous registration of compounds separated by gas chromatography with a chemical detector (Flame Ionisation Detector, FID) and a biological detector (Electro Antennographic Detector EAD). The drawing shows separation of a pheromone sample from a cone pyralid Dioryctria abietella, monitored with a male antenna. The arrow indicates the main pheromone component, hardly detected by the FID but clearly indicated by the EAD! (From Löfstedt et al. 1983).

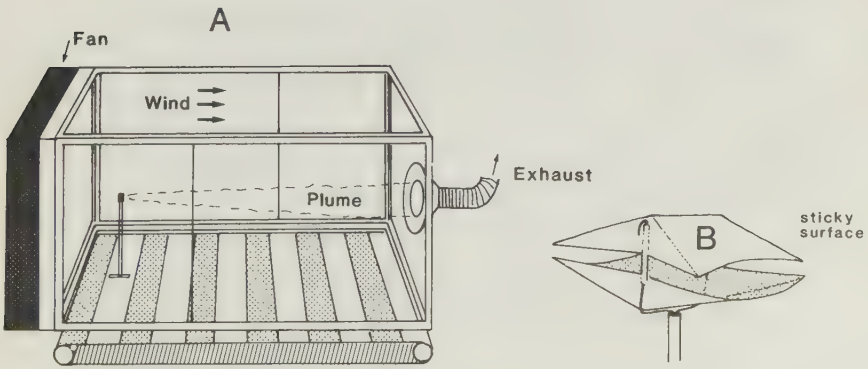

Figure 4: In the laboratory a flight tunnel (A) is used for behavioural tests. Baits to be tested are presented upwind, moths are introduced at the opposite end, and the male behavioural response is recorded. In the field the study of attraction to sticky traps, baited with attractive lures is a commonly used technique (B).

testing of experimental hypotheses then becomes complementary to the more feasible laboratory experiments.

THE SEX PHEROMONE COMPONENTS OF THE TURNIP MOTH

The pheromone gland of the female turnip moth Agrotis segetum contains about 20 alcohols and acetates of possible relevance to pheromone communication. (Paper I, Paper VI, Lanne et al. 1984). This set of substances is characteristic of compounds identified as noctuid pheromones (Steck et al. 1983). According to the present concept of moth pheromone biosynthesis (Bjostad & Roelofs 1983, Roelofs & Brown 1982), most of them can be biosynthetically accounted for by the combined action of an enzyme introducing a double-bond between the 11th and 12th carbon (a delta 11-desaturase) and a chain-shortening enzyme (Fig. 5).

The only unsaturated acetate that does not fit into the delta 11-desaturase framework is Z5-14:OAc. This compound is probably derived from oleic acid, (Z)-9-octadecenoic acid, biosynthesized by the interaction of the ubiquitous delta 9-desaturase with stearic acid. The presence of Z7-16:acyl moities as a probable chain-shortening intermediate lend support to this idea (Paper VI). So far Z5-14:OAc has no proven behavioural activity in the turnip moth. However, the closely related A. exclamationis uses a precise mixture of Z5-14:OAc and Z9-14:OAc in its pheromone (Bestmann et al. 1980). This suggests how adaptive radiation might take place within a genus. A.segetum and A.exclamationis have the same basic biosynthetic potential, manifested in the occurrence of the same pheromone precursors (Paper VI and Löfstedt unpublished) in both species. However, these are used in different ways to produce the species specific pheromones.

In the Japanese equivalent of the European turnip moth, Agrotis segetum (fucosa) the pheromone is reported to consist of Z5-10:OAc and Z7-10:OAc (Wakamura 1978, 1980). This is qualitatively different from the European mixture of Z5-10:OAc, Z7-12:OAc and Z9-14:OAc (Paper I). Z7-10:OAc should most probably originate from the interaction of the delta 11-desaturase with tetradecanoic acid, then producing Z7-10:OAc by chain-shortening. There is no evidence for this pathway in the European moths (Paper VI).

Two compounds, E5-12:OAc and Z8-12:OAc, reported by Arn et al. (1980) to occur in turnip moth gland extracts, were not found in the present studies. Probably, they should be considered as contaminants or misidentifications as they can not be derived from the biosynthetic pathways active in A. segetum.

For the adaptationist, it should be important to consider biosynthetic constraints on pheromone evolution. The array of enzymes available for pheromone biosynthesis, sets the limits for evolution of chemical communication within a taxon.

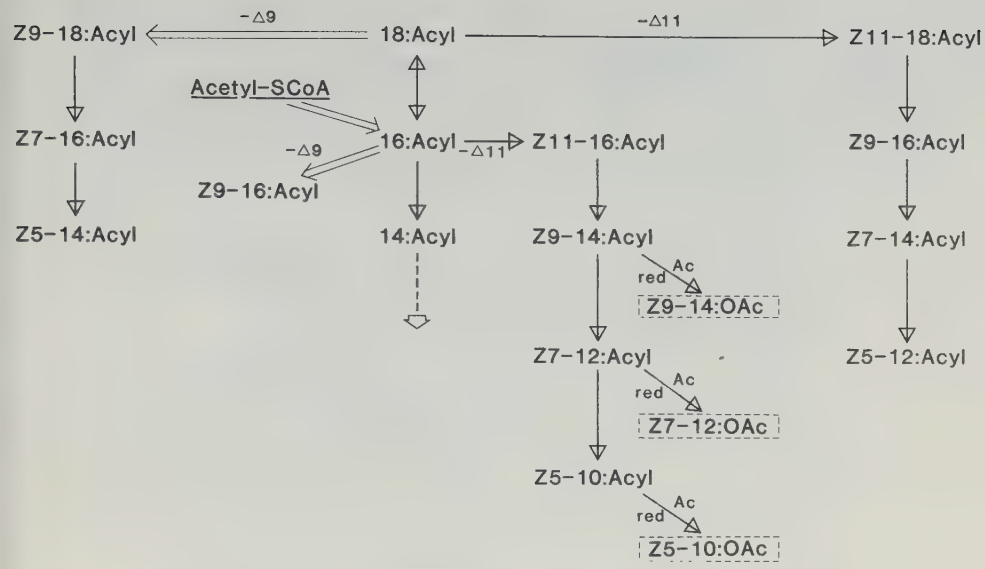

Figure 5. Postulated pathways for the biosynthesis of turnip moth pheromone components. The saturated fatty acid with 16 carbons (palmitic acid) is produced by the ordinary cell fatty acid biosynthesis. This starting material can be chain-elongated, chain-shortened or desaturated (double bond introduced). The pheromone components (encircled) have a common origin: A double bond is introduced by an unusual enzyme in the position between the 11th and 12th carbon atom by interaction with palmitic acid. The Z11-16:acyl intermediate is then chain-shortened in several steps to give rise to the fatty acids corresponding to the acetates in the pheromones. The acids are probably reduced to alcohols before the final acetylation.

BEHAVIOURAL AND ELECTROPHYSIOLOGICAL ACTIVITY OF PHEROMONE COMPONENTS

Not all of the acetates and alcohols isolated from the female pheromone gland are necessarily associated with behavioural activity. They might also be biosynthetic intermediates or by-products. Which of these compounds are behaviourally active and how are they perceived by the male?

The behavioural activity of the homologous acetates Z5-10:OAc, Z7-12:OAc and Z9-14:OAc was confirmed in a flight tunnel assay as well as in the field (Paper III). GC-EAD and a primitive tube olfactometer assay (Paper I) had drawn the attention to 10:OAc as a gland constituent with possible biological activity. However, this compound could be subtracted from the 4-component blend without any loss of activity (Paper III). Furthermore refined electrophysiological detection, i.e. continuous single sensillum recording, at the outlet of the gas chromato-- graph, did not indicate any activity in the region of

10:OAc (Paper II). The combined GC-single sensillum instrumentation was developed to improve the signal to noise ratio in GC-electrophysiology recordings and to be able to identify the receptor involved with the electrophysiological activity. In one GC-single sensillum experiment, however, information can be obtained on only one type of sensillum, whereas GC-EAG detection provides information from many types of olfactory sensilla. The GC-single sensillum technique is a valuable supplement but not a substitute for GC-EAD.

Z5-10:OAc, Z7-12:OAc and Z5-10:OH were the only gland constituents with significant EAG-activity. Nevertheless, Z9-14:OAc seemed to be necessary for good behavioural activity of a synthetic pheromone (Paper I and III). It was hypothesized that Z9-14:OAc was received by specialized cells on the male antenna, which occur in very low numbers. Recording from hundreds of sensilla indeed confirmed this hypothesis (Paper I and VI). Only 2% of the male sensilla trichodea contained receptors for Z9-14:OAc. The sensilla containing the Z5-10:OAc receptor also contained a receptor activated by Z5-10:OH and Z8-12:OAc. Z5-10:OH was identified from gland extracts of A.segetum but added to the active mixture of acetates it reduced the attraction of males to traps. Thus the amount of Z5-10:OH isolated from female glands might be an extraction artifact. The alcohols might be pheromone precursors for the final acetylation before release.

Non-pheromone chemicals that negatively influence attraction have been referred to as "inhibitors", "maskers" or "anti-attractants" in various contexts (see Cardé 1976). It is interesting that Z8-12:OAc, stimulating the same receptor as Z5-10:OH also reduces trap-catches (Paper III, Arn et al. 1980). In Paper III it is suggested that the mode of action of Z8-12:OAc on male A.segetum pheromone behaviour is mainly arrestment of upwind flight. In contrast another isomer, the Z6-12:OAc, did not have a negative effect but rather increased the attraction. Obviously a negative activity can not be assigned to every pheromone analogue.

The most active synthetic blend tested in the flight tunnel was inferior to female glands. Further, males responded faster to the glands than to any of the synthetic baits. The reason for the weaker response could be missing trace components or a suboptimal ratio of the three homologous acetates. (Paper III, IV, V and Arn et al. 1983).

In conclusion at least three acetates found in the female are behaviourally active (Fig. 6), but none of them can be assigned a specific function. All of them seem to affect all behavioural steps in the process of male attraction to the female. Each of the compounds is received by a set of specialized receptor cells on the male antenna, but the number of receptors for one of the compounds is very low (only 2 % of the cells). Identification of receptors associated with trace pheromone components might require recordings from not only hundreds but thousands of sensilla (Van Der Pers & Löfstedt, in press).

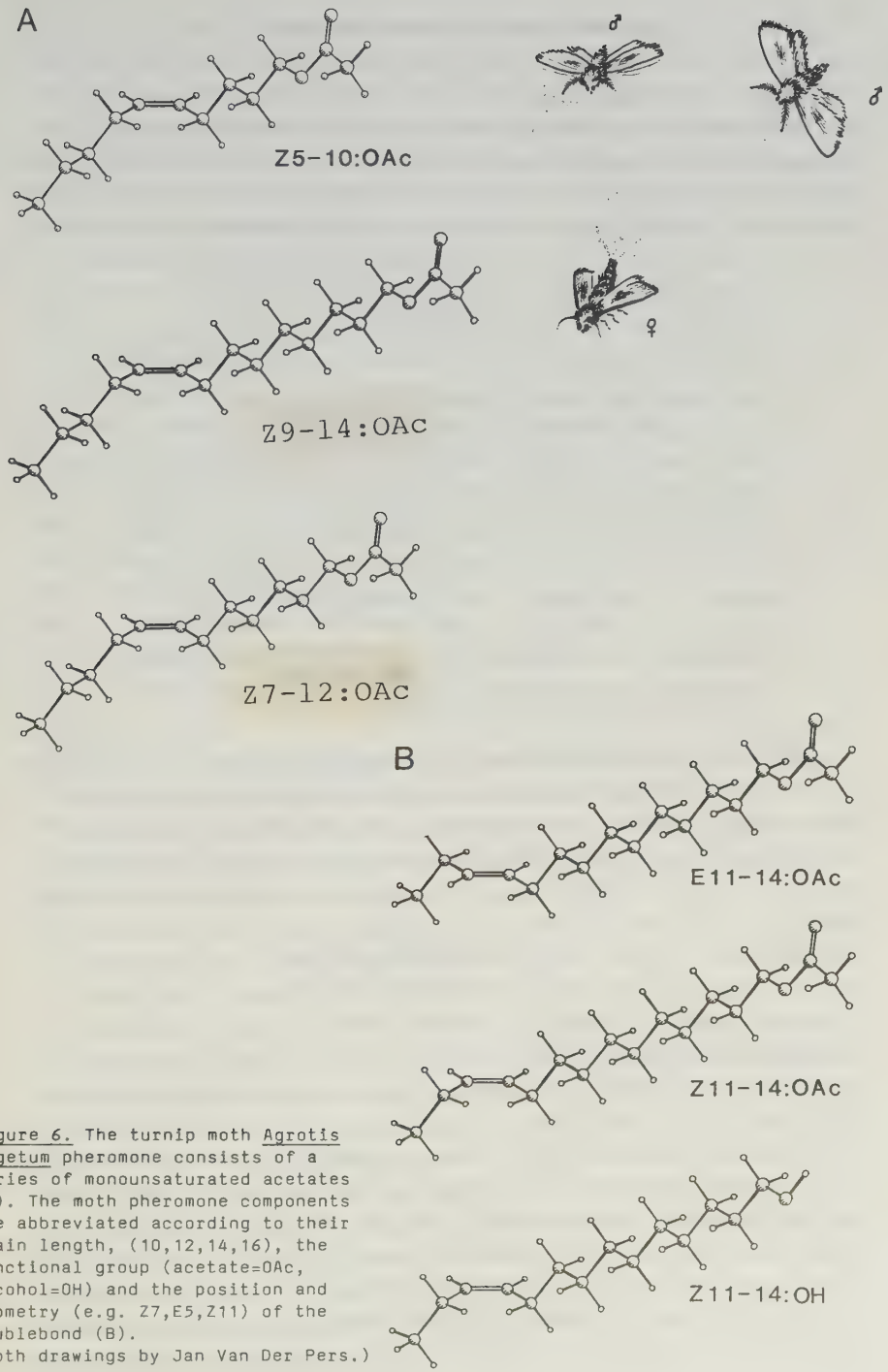

Figure 6. The turnip moth Agrotis segetum pheromone consists of a series of monounsaturated acetates (A). The moth pheromone components are abbreviated according to their chain length, (10,12,14,16), the functional group (acetate=OAc, alcohol=OH) and the position and geometry (e.g. Z7,E5,Z11) of the doublebond (B).
(Moth drawings by Jan Van Der Pers.)

PHEROMONE VARIATION AMONG INDIVIDUALS

The principal mechanism of Darwin's theory of evolution is natural selection acting on individual differences. What is the magnitude of variation in the pheromone production within a population? How does the pheromone quality of a single female affect her ability to attract a male and reproduce? What are the evolutionary implications of variation in pheromones between individuals?

> Analysis of individual female turnip moths by gas chromatography with flame ionization detection is possible, even though the titre of the major pheromone component (Z7-12:OAc) is as low as 0.5 - 3 ng. Highest sensitivity is achieved by the use of redistilled solvents, microsurgery to isolate the gland tissue only, by the use of hydrogen as the carrier gas and by separation on state-ofthe-art fused silica columns coated with immobilized stationary phase (Paper VI) . However, analysis of individuals by means of GC-FID remains sensitive to contamination. Combining the gas chromatograph with a mass spectrometer operated in the selected ion monitoring mode (SIM) provides a very selective means of identifying and quantifying moth pheromones (Paper IV). For acetates limits of detection and quantification were good with chemical ionization using ammonia as the reactant gas, as well as with electron impact ionization monitoring m/z 61 and 82. A simple but precise method for quantification using deuterated internal standards and chemical ionization with ammonia, was described . A significant decrease in the pheromone titre of individual females as a result of mating was demonstrated, using this method.

In Paper V individual variation in pheromone production in two strains of turnip moths was quantified. One strain was inbred for more than 30 generations and the other was first to third generation offspring from insects collected as pupae in the field. The analyses demonstrated a considerable variation between individual females in the proportion of the pheromone compounds. The high variation indicates a pheromone system very different from the redbanded leafroller moth, for which Miller & Roelofs (1980) found a precisely regulated ratio between the pheromone components. Even though Z7-12:OAc generally was the most abundant pheromone component there were actually several females where either Z5-10:OAc or Z9-14:OAc was the major constituent. Differences in pheromone composition between the inbred and the wild strain were not statistically significant.

To estimate mating success of individual females in relation to the composition of their pheromones a field experiment was performed. A series of synthetic pheromone blends was made up to cover the range of variation in pheromone composition observed in females. The most potent blend attracted twice as many males as the least attractive blend (Paper V). Does this reflect real differences in female attraction of males? Theoretically there should be low additive genetic variance in characters (such as sexual attractants) closely

related to fitness (Maynard Smith 1978). A solution to the paradox could be that the female variation documented is not genetically determined. The pheromone composition could vary with, for instance, the age of the female or the time of day. However, more likely would be that the range of variation in attractivity observed in this study is evolutionary insignificant. Males can mate several times and all females that produce at least a minimum amount of the pheromone components might eventually become mated.

Assuming that pheromones produced by moths are given off by passive diffusion, blend components with different chain length (or different functional moieties, e.g. alcohols and acetates) would evaporate at slightly different rates from the gland surface at different environmental temperatures. Hence the composition of the pheromone released from a calling female would vary with temperature. Cardé and Baker (1984) have suggested that this is the raison d'étre of several divergent blends in moths, precluding the use of such compounds in very precise ratios. But this is hardly true in case of the turnip moth, even though this species uses pheromone components of three different chain-lengths. Even extreme temperature changes will only cause small changes in the pheromone composition according to theoretical calculations (Paper V). For example a temperature shift from $5^{o}C$ to $20^{o}C$ will at most shift the pheromone composition by 3 %. This is far less than the range of female produced ratios and the range of blends attractive to males. There is probably an evolutionary cost associated with the production of precise pheromone blends. Then a precisely regulated pheromone will evolve only when there is a strong selection in favour of species specificity achieved by this means. In the turnip moth the three homologous unsaturated acetates might give enough specificity on a qualitative basis such that a precise regulation of ratios is not critical.

PHEROMONE DIALECTS IN EUROPE

The pheromone composition in a population is sensitive to various types of selection. If other selection pressures (as interactions with other species) are missing, stabilizing selection (i.e. selection for the population norm) should prevail. Stabilizing selection favours males sensitive to the pheromone released by the majority of females, and those females emitting the blend to which males are most sensitive (Cardé and Baker 1984). Male turnip moths in different European countries seem to be differentially tuned to blends of the pheromone components. These male differences should presumably be correlated with differences in the female pheromone production, and if so, be regarded as evidence for different dialects in the chemical language of the moths.

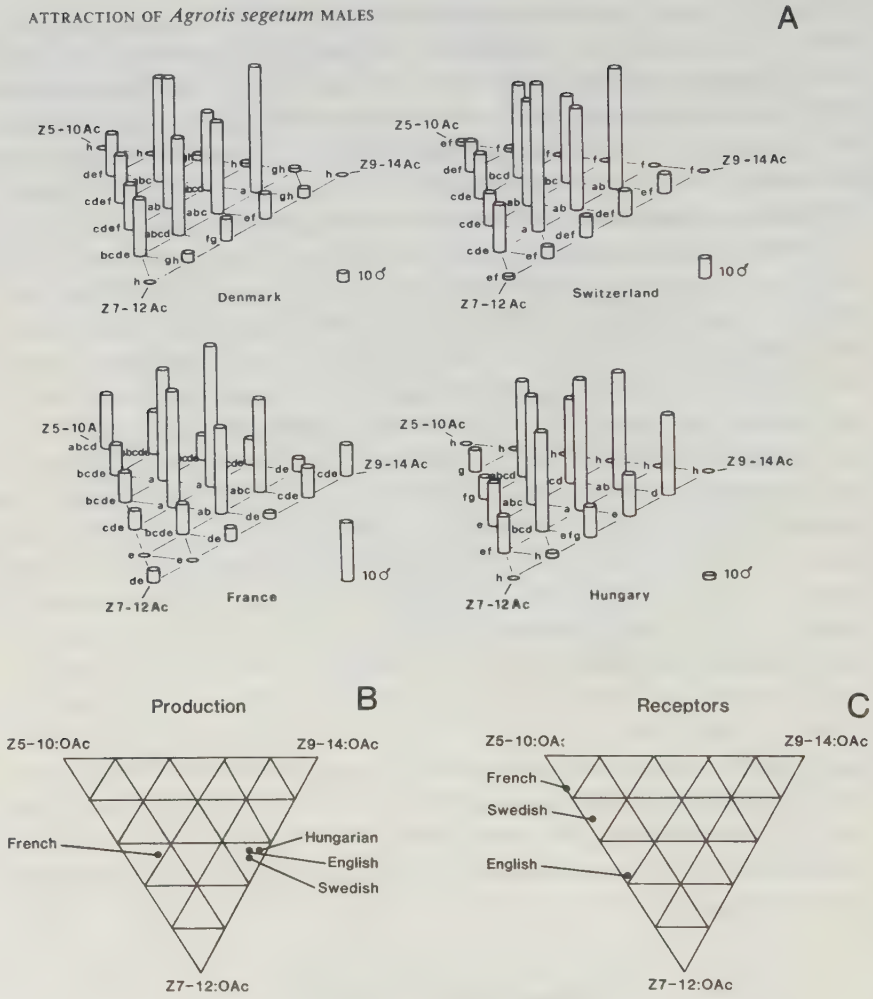

Figure 7. Dialects in the chemical language of the turnip moth Agrotis segetum. Field tests with Z5-10:OAc, Z7-12:OAc, Z9-14:OAc and their binary and ternary mixtures in four European countries (A) (from Arn et al. 1983). Relative female production of the three pheromone components in turnip moth cultures from four European countries (B) and relative frequency of pheromone receptors on the antennae of males from three different countries (C) (data from Paper VI). There is an interesting correlation between the abberant response of male French turnip moths to pure Z5-10:OAc, the higher frequency of Z5-10:OAc receptors and the higher relative production of Z5-10:OAc in the French insects, indicating a French pheromone dialect.

Arn et al. (1983) performed a field screening test of different combinations and different ratios of the pheromone components Z5-10:OAc, Z7-12:OAc and Z9-14:OAc. Their experiment indicated geographical differences in the male

response profiles, e.g. French A.segetum males were the only ones to be attracted by Z5-10:OAc as a pure compound. Paper VI confirms the existence of pheromone dialects in European populations of the turnip moth A.segetum. The aberrant male response of French turnip moths to pure Z5-10:OAc reported by Arn et al. (1983) is correlated with a higher frequency of Z5-10:OAc receptors on the male antennae and a higher relative titre of Z5-10:OAc in the French females (Fig. 7). Furthermore our analysis of the postulated pheromone precursors indicates a possible biosynthetic explanation for the observed difference in female pheromone production.

The apparent correlation between pheromone production, pheromone perception and behavioural response in A.segetum is interesting. Alexander (1962) and O'Donald (1962) suggested from a theoretical point of view that species-specific features of communication systems should be inherited as blocks of co-adapted gene complexes or supergenes. Whether such a gene complex accounts for the pattern observed in the turnip moth remains to be investigated.

Pheromone dialects in the turnip moth could be the outcome of random genetic changes or the result of adaptation to different interspecific interactions within the species range of distribution. If character displacement or character release could be demonstrated, this would lend support to the adaptive explanation;

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SEX PHEROMONE COMPONENTS OF THE TURNIP MOTH, *Agrotis segetum*[1]: Chemical Identification, Electrophysiological Evaluation and Behavioral Activity[2]

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Abstract—Analysis of female abdominal tips of *Agrotis segetum* by means of GC-MS showed the presence of 13 aliphatic acetates and alcohols. (*Z*)-7-Dodecenyl acetate was found to be the main component in the extracts at amounts of about 1 ng/female. (*Z*)-9-Tetradecenyl acetate and (*Z*)-7-dodecenol were present to the extent of 49 and 19%, respectively, of the main component. Minor components could be identified as decyl acetate, (*Z*)-5-decenyl acetate, dodecyl acetate, (*Z*)-9-dodecenyl acetate, tetradecyl acetate, a tetradecenyl acetate, hexadecyl acetate, a hexadecenyl acetate, (*Z*)-5-decenol, and (*Z*)-9-tetradecenol. The presence and biological activity of decyl acetate, (*Z*)-5-decenyl acetate, and (*Z*)-7-dodecenyl acetate in the extracts could be detected by GC-EAD. Tested by EAG (*Z*)-5-decenyl acetate evoked the highest response among pheromone candidates, followed by (*E*)-5-decenyl acetate and (*Z*)-7-dodecenyl acetate. Single-cell recordings from 100 male antennal sensilla trichodea revealed receptor cells highly sensitive to (*Z*)-5-decenyl, (*Z*)-7-dodecenyl, (*Z*)-8-dodecenyl, and (*Z*)-9-tetradecenyl acetate as well as (*Z*)-5-decenol. The (*Z*)-5-decenyl, (*Z*)-7-dodecenyl, and (*Z*)-9-tetradecenyl acetate receptors were activated significantly also by female extracts. When tested in a tube olfactometer, a blend of decyl, (*Z*)-5-decenyl, (*Z*)-7-dodecenyl, and (*Z*)-9-tetradecenyl acetate evoked the same male response as did female glands.

[1]Schiff., Lepidoptera: Noctuidae.
[2]This study was made within the Swedish project "Odour Signals for Control of Pest Insects."

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Tested in the field, this blend was more attractive than virgin females. Other authors previously reported many of the compounds identified in the present study. However, both quantitative and qualitative discrepancies exist among the various investigations, possibly due to the existence of geographical races.

Key Words—*Agrotis segetum*, turnip moth, Lepidoptera, Noctuidae, sex pheromone, straight-chain acetates, single-cell recordings, electroantennography, behavior, gas chromatography–mass spectrometry, entrainment.

INTRODUCTION

Multicomponent sex pheromones seem to be more the rule than the exception in moths (Silverstein and Young, 1976; Tamaki, 1979). The sex pheromone of the turnip moth *Agrotis segetum* (Schiff.) (Lep., Noctuidae) is a recent example. Bestmann et al. (1978) reported (*Z*)-5-decenyl acetate (*Z*5–10:Ac) to be a sex attractant of *A. segetum*. Tóth et al. (1980) described the pheromone of *A. segetum* as composed of a 4:1 mixture of *Z*7–12:Ac and *Z*9–14:Ac, while Arn and coworkers (1980) reported the pheromone of the species to consist of a blend of *Z*5–10:Ac, *Z*7–12:Ac, *Z*9–12:Ac, *E*5–12:Ac, and 12:Ac. However, the behavioral significance of the latter three compounds was unclear. Another compound, *Z*8–12:Ac, was identified from the female pheromone gland, but it was assigned an inhibitory effect on male attraction.

In addition to the studies on European turnip moths, Wakamura (1978, 1980) identified a mixture of *Z*5–10:Ac and *Z*7–10:Ac as the pheromone of the Japanese *Agrotis fucosa*, which was synonymized recently by taxonomists with *A. segetum* (S. Wakamura, personal communication).

The present study was initiated in 1980 when we failed to trap *A. segetum* in the field with *Z*5–10:Ac, the sex attractant reported by Bestmann et al. (1978). We then started a detailed GC-MS examination of the pheromone of *A. segetum*. The chemical studies were accompanied by investigations of the electrophysiological and behavioral significance of the identified compounds.

METHODS AND MATERIALS

Insect Material. A. segetum moths were obtained from a culture permanently maintained at the Ecology Building, University of Lund, and based on insects originating from southern Sweden and Denmark. The culture had not been fortified with wild insects since 1978. Thus insects used for this study were inbred for about 20 generations. Larvae were reared on a Hinks and Byers' (1976) diet, using potato instead of pea beans. The culture was maintained on a 17-hr light–7-hr dark cycle with photophase temperature

23°C and scotophase temperature 16°C for both pupae and adults. Larvae were kept under the same light regime but at a temperature of 26°C.

The insects were sexed as pupae and placed in separate compartments. After emergence, 1–3 adults were kept in 250-ml plastic jars and fed a 5% honey solution. Females used for preparation of extracts were fed a 5% sucrose solution.

Observation of Calling Behavior. Female moths used for studies of calling behavior were placed individually in 250-ml plastic jars covered with a nylon screen. Red darkroom lamps (ca. 10 lux at moths) were used to permit observation during the scotophase. The insects were observed during 10 nights after emergence. To determine the calling period, observations were made during the entire dark period at 30-min intervals, and the number of calling females was recorded. Once the calling period had been ascertained observations were made from 1 hr before to 1 hr after calling.

Females were regarded as calling when they clung to the walls or to the screen cover and exposed their ovipositors. Their pheromone glands were then readily visible.

The mean time of day of calling activity (T) was calculated as follows:

$$T = \frac{\Sigma[(\text{time}) \times (\text{No. of females calling at that time})]}{\text{Total No. of callings recorded}}$$

The time is measured as the number of hours into the dark period.

Preparation of Extracts. Extracts were prepared from 2- to 5-day-old females within a 2-hr period of maximum calling activity (see Figure 1). The abdominal tip was stripped off immediately anterior to the pheromone gland with a pair of forceps (Otto et al., 1976). Before immersion in a sharply coned dissection vial containing 100–500 μl solvent (pentane or hexane), gut contents and hemolymph were removed by adsorption on filter paper. The dissection vial was kept at −20° C during preparation and afterwards stored in the dark at −20° C. The solvent was recovered with a syringe 24 hr following the last preparation. It was stored in a sealed ampoule at −20° C until analysis.

Entrainment. Female effluvium was examined using charcoal filters similar to those described by Grob and Zürcher (1976). One to three moths were placed in a glass entrainment chamber (250 ml) before the beginning of the dark period. Air (charcoal-filtered and humidified) was passed through a chamber at a rate of 1.3 liters/min. Volatiles were entrained on the same filters for 5 hr starting from 1 hr into the dark period. Entrainment from one female obtained in this way was called "one female calling night."

Adsorbed material was extracted with three portions of carbon disulfide (15 + 10 + 10 μl).

GC-MC. Gas chromatographic–mass spectrometric analyses were performed on a Finnigan 4021 GC-MS equipped with an Incos data system.

Pentane and hexane extracts of 20–80 female abdominal tips were concentrated to approximately 20 μl by careful heating. Approximately a fourth of this sample was injected, splitless, on a WCOT capillary column (60 m × 0.24–0.30 mm ID) coated with Superox FA (Alltech Assoc.). The linear helium flow through the column was 26 cm/sec at 60°C. The injector temperature was 210°C. The split valve was opened 0.5 min after injection. Column temperature was maintained at 60°C for 5 min following injection, and then heated to 220°C using a program of 10°C/min.

In certain analyses 10:Ac, 12:Ac, 14:Ac, 16:Ac, and 18:Ac were added as references for quantifications or for the calculation of retention values relative to the straight-chain acetates [equivalent chain length (ECL) values]. ECL values from the same substance never differed more than two ECL units in subsequent analyses.

GC-FID and GC-EAD. Gas chromatographic analyses were performed on a Hewlett Packard 5835 gas chromatograph. An adjustable micro needle-valve effluent split (Scientific Glass Engineering, Inc.) was inserted between the column and the detector to permit simultaneous flame ionization detection (FID) and electroantennographic detection (EAD) (Arn et al., 1975). A split ratio of 4:1 was used.

Aliquots of sample (1–5 μl) were injected, splitless, on a WCOT fused silica capillary column (20 m × 0.19 mm ID) coated with SP 1000 (Supelco, Inc.). The nitrogen flow through the column at 60°C was 13 cm/sec. The injector temperature was 250°C. The split valve was opened 0.5 min after injection. Column temperature was maintained at 60°C for 5 min following injection, and then raised to 200°C using a program of 8°C/min. Under these conditions, standard samples of most long-chain olefinic acetates could be resolved. The chromatographic conditions allowed flame ionization detection of ca. 200 pg. Although no actual quantification was undertaken, electroantennographic detection on the order of 1 pg of Z5–10:Ac using a male *A. segetum* antenna as detector was possible.

Ozonolysis. One extract obtained from approximately 50 females was fractionated according to chain length of acetates and alcohols on a Carlo Erba 4160 GC equipped with a Superox FA SCOT column. Ozonolysis according to Beroza and Bierl (1967) was performed on the fractions. Separation and identification of the ozonolysis products were obtained by GC-MS.

Electrophysiology. Electroantennogram (EAG) and single-cell experiments were performed on excised antennae of male *A. segetum* moths. The experimental arrangement used in the EAG measurements was essentially the same as described by Van Der Pers (1981). Single-cell recordings were made using the tip recording technique as applied by Van Der Pers and Den Otter (1978).

In both EAG and single-cell experiments the odor sources were

disposable syringes containing a piece of filter paper (13 × 13 mm) onto which 1 μg of a given test substance had been pipetted. The test substances are given in Figure 4.

The maximum EAG amplitude was used as a measure of response in the EAG experiments and expressed as a percentage relative to the reference response.

Tube Olfactometry. Male behavioral response to different stimuli was studied in a tube olfactometer similar to that described by Sower et al. (1973). The glass tube was 45 mm in diameter and 450 mm long with a glass filter in the upwind end and a nylon screen at the outlet. Filtered, compressed air was delivered to the tube at a rate of 0.3 m/sec.

The tube was lighted uniformly from below with red light (650 nm peak emission, 3 lux) through white transluscent Perspex. A male, 2–4 nights old, was introduced downwind in the tube at least 10 min before the onset of the stimulation. The tube was connected to the air supply 2 min prior to stimulation.

Stimuli were inserted 25 mm from the upwind end of the tube. Female glands to be tested for activity were excised from calling females and held in the air stream with a pair of forceps. Synthetic chemicals were applied in hexane (1 μl) to a glass rod applicator, which was inserted into the tube as soon as the solvent had evaporated. Male activity was observed during 2 min after the stimulation was started. The frequencies of the following male behavioral steps (cf. Baker et al., 1976) were recorded on a tape recorder: (1) orientation into the 5-cm upwind end of the tube; (2) wing-fanning while walking or flying short distances; (3) extension of their genital claspers; and (4) clinging to the applicator, trying to copulate with it.

The tubes were successively washed with ethanol, acetone, and heptane and subsequently heated to 170°C for at least 1 hr between experiments.

Field Attractancy Test. Between June 26 and July 14, 1981, field tests were conducted close to Lund (southern Sweden), using traps similar to those described by Kärnestam (1979). The traps consisted of a plastic tube (8 cm diam. × 40 cm long) attached to a wind vane which was free to rotate on a pole 1.2 m above ground. A conical metal screen on each end directed males inside the tube but prevented their escape. Fourteen pairs of traps were spaced at least 60 m from each other in lettuce, potato, and carrot fields. The distance between traps within each pair was 4 m. One trap of a pair was loaded with a newly emerged virgin female from a laboratory culture and the other with a synthetic pheromone blend. When baited with a virgin female, a metal screen cage inside the tube contained the insect. The insect was fed sucrose solution and replaced every week. When baited with synthetic chemicals, the insect cage was changed for a 1-ml polyethylene capsule (36 × 8 mm, 1.5 mm wall thickness). The capsule was loaded with chemicals dissolved in pentane/hexane. The solvent was allowed to evaporate before the capsule was closed.

Synthetic baits were not changed during the experimental period. Traps were checked every third or fourth day.

Chemicals. Z4–10:Ac, E5–10:Ac, Z5–10:Ac, Z6–10:Ac, E7–12:Ac, Z7–12:Ac, and Z9–14:Ac were synthesized via the corresponding alkynes, which were hydrogenated with Lindlar catalyst and Na/NH_3 for *Z* and *E* isomers, respectively. After purification (Houx et al., 1974), these compounds were more than 99.9% pure with respect to positional and geometrical isomers.

Other compounds used were commercially available or gifts from several laboratories.

RESULTS

Female Calling Rhythm. Female calling activity was at a maximum 4.5 hr after the beginning of the dark period. The mean time of calling was 3.9 ± 0.9 hr ($N = 86$) into the dark period (Figure 1). Differences in the percentage of females calling and diurnal rhythm of calling between the second and the fifth night after emergence were minor.

GC-MS Analyses and Ozonolysis. A typical reconstructed total ion chromatogram from an analysis of 9 female equivalents is shown in figure 2A. Figure 2B shows the corresponding mass chromatograms (extracted ion

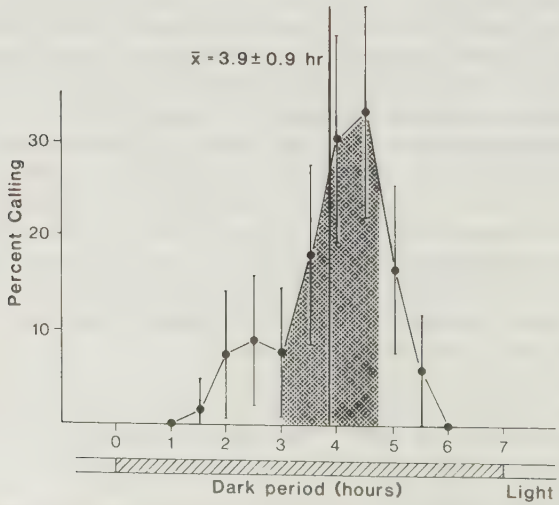

FIG. 1. Percent calling by female *Agrotis segetum* on their fifth night after emergence as calculated from observations every 30 min ($N = 66$). Vertical lines indicate 95% confidence intervals. $\bar{X}$ is the mean time of day of calling (±SD, shaded area).

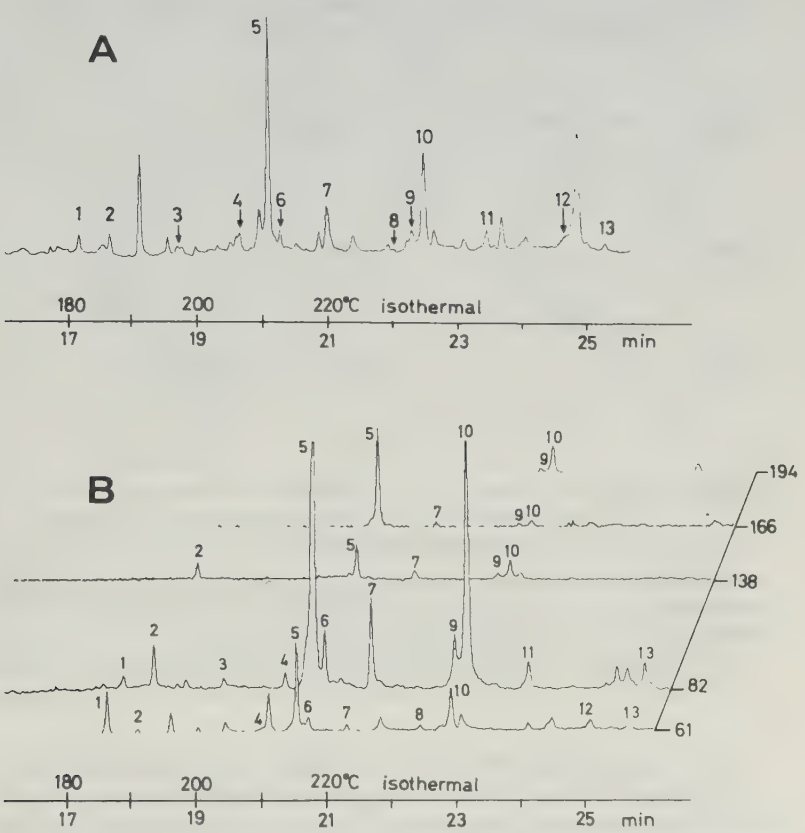

FIG. 2. (A) Total ion chromatogram, m/z 30–31, 33–300 and (B) mass chromatograms (extracted ion current profiles) of m/z 61, 82, 138, 166, and 194 of an extract corresponding to 9 female *A. segetum* abdominal tips. m/z 61 is a typical fragment of acetates, and m/z 82 indicates unsaturated straight-chain compounds. m/z 138, 166, and 194 correspond to M^+-CH_3COOH of decenyl, dodecenyl, and tetradecenyl acetate respectively, and to M^+-H_2O of decenol, dodecenol, and tetradecenol. Peak numbers refer to compounds listed in Table 1.

current profiles) for some fragments of diagnostic value. Compounds found with chemical structure similar to moth pheromones identified earlier can be divided into the following three groups:

1. Saturated Acetates. The compounds corresponding to peaks 1, 4, 8, and 12 were identified as 10:Ac, 12:Ac, 14:Ac, and 16:Ac, respectively, from their mass spectra and retention values.

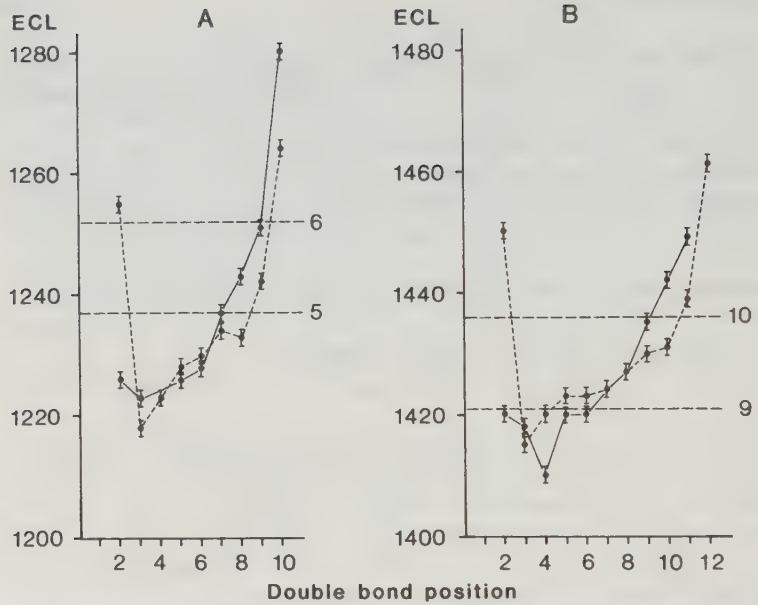

FIG. 3. Variation in equivalent chain length (ECL) with the position of the double bond; dodecyl acetate = 1200, tetradecyl acetate = 1400, on a superox FA WCOT capillary column. (A) dodecenyl acetates, (B) tetradecenyl acetates; – – – – *E* isomers, ——— *Z* isomers. Vertical lines indicate maximum spread (±2 ECL units). Compounds 5, 6, 9, and 10 refer to substances isolated from *A. segetum* females.

2. Monounsaturated Acetates. From their mass spectra the following assignments were made: compound 2 was a decenyl acetate, compounds 5 and 6 dodecenyl acetates, compounds 9 and 10 tetradecenyl acetates, and compound 13 a hexadecenyl acetate.

The calculated ECL (1037) of compound 2 is within the range of both *E*5–10:Ac and *Z*5–10:Ac. These two isomers were not separated by GC. Other decenyl acetate references available were *Z*4 (1020), *Z*6 (1043), *E*7 (1045), and *Z*7 (1052).

The ECL of compound 5 (1237) corresponds to that of synthetic *Z*7–12:Ac (Figure 3A). Compound 6 (1252) could be either the *E*2 or the *Z*9 isomer. However, the mass spectra of these compounds differ: *E*2 shows significantly less abundant fragments in the high mass range. Thus compound 6 was identified as *Z*9–12:Ac.

Concerning the tetradecenyl acetates, compound 9 could probably be any of the following isomers: *E*4, *E*5, *E*6, *Z*5, or *Z*6. The calculated ECL (1436) of peak 10 is identical to that of synthetic *Z*9–14:Ac (Figure 3B). This assignment was confirmed by ozonolysis. On the other hand, the isolated

TABLE 1. AMOUNTS OF SUBSTANCES RELATIVE TO AMOUNT OF *Z*7-12:Ac ISOLATED FROM *Agrotis segetum* FEMALE ABDOMINAL TIP EXTRACTS

Assignment	No.	Relative amount
10 : Ac	1	8
*Z*5-10 : Ac	2	6
*Z*5-10 : OH	3	3
12 : Ac	4	5
*Z*7-12 : Ac	5	100[a]
*Z*9-12 : Ac	6	7
*Z*7-12 : OH	7	19
14 : Ac	8	1
Z/*E*?14 : Ac[b]	9	6
*Z*9-14 : Ac	10	49
*Z*9-14 : OH	11	8
16 : Ac	12	2
Z/*E*?16 : Ac[c]	13	3

[a] The amount of *Z*7-12 : Ac isolated per female was approximately 1 ng.
[b] This tetradecenyl acetate isomer was probably one of the following: *E*4, *E*5, *E*6, *Z*5, or *Z*6 (see text).
[c] Double-bond isomerism was not determined.

amount of compound 9 (about 3 ng from 50 females Table 1) was too low for ozonolysis.

The isomerism of the hexadecenyl acetate was not determined.

3. Monounsaturated Alcohols. The mass spectra of peaks 3 (1122), 7 (1313), and 11 (1509) correspond to those of a decenol, a dodecenol, and a tetradecenol, respectively. These ECL values correspond with those of synthetic *Z*5-10:OH, *Z*7-12:OH, and *Z*9-14:OH. However, only a limited number of references was available (one decenol, five dodecenols, four tetradecenols) and correspondence with other isomers cannot be ruled out.

Simultaneous GC-FID and GC-EAD of Female Extracts and Entrained Material. GC-EAD of female extracts with a male *A. segetum* antenna as the detector gave an EAD chromatogram with three reproducible peaks. The retention times of these compounds corresponded to the retention times of synthetic 10:Ac, *Z*5-10:Ac, and *Z*7-12:Ac, respectively. The *A. segetum* antenna did not give a significant response to *Z*9-14:Ac which was shown by GC-MS and ozonolysis analysis to be one of the major compounds in the female gland extract. However, when a male *Adoxophyes orana* (*F.v.R.*) antenna was used as the detector, the presence of *Z*9-14:Ac in the *A. segetum* female extract was confirmed. The antenna of *A. orana* is known to be highly sensitive to *Z*9- and *Z*11-14:Ac (Den Otter, 1977). With this antenna, a strong

EAD response (2 mV) was obtained from the extract of an *A. segetum* female gland at the same retention time as that of synthetic *Z*9–14:Ac.

A sample containing the entrained volatiles from half a female calling night gave rise to an EAD chromatogram similar to that of a female gland extract. The decyl acetate and (*Z*)-5-decenyl acetate peaks were of the same magnitude as in several analyses of gland extracts, while the *Z*7–12:Ac was smaller but still significant. The FID signal of *Z*7–12:Ac corresponded to somewhat less than 1 ng/female calling night. In a single experiment using a *A. orana* antenna it was not possible to detect *Z*9–14:Ac in the entrained volatiles.

Electrophysiology. Several compounds identified by means of GC-MS or GC-EAD in this study or mentioned earlier in the literature (Arn et al., 1980; Wakamura, 1978) can be considered as *A. segetum* pheromone candidates. Of these *Z*5–10:Ac showed the highest EAG activity (Figure 4): two times higher than *E*5–10:Ac. The following compounds elicited lower EAG activity: 10:Ac, *Z*5–10:OH, *Z*7–12:Ac, and *Z*8–12:Ac. However, the responses were significantly higher than those obtained from the other compounds tested.

The single-cell responses of 100 sensilla trichodea to the same set of substances (with the exception of *E*5–10:Ac) are summarized in Table 2. Receptor cells very sensitive to *Z*5–10:Ac, *Z*7–12:Ac, *Z*9–14:Ac, *Z*5–10:OH, and *Z*8–12:Ac were present. The first three compounds activated cells

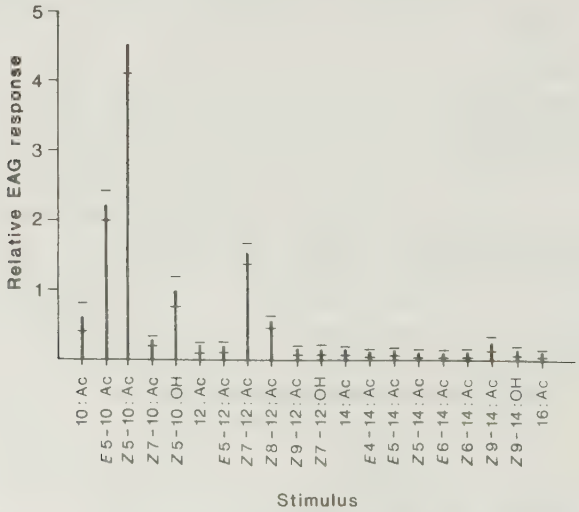

FIG. 4. Relative EAG responses to compounds tested as *A. segetum* pheromone component candidates.

TABLE 2. SINGLE-CELL SCREENING OF 100 SENSILLA TRICHODEA ON MALE *A. segetum* ANTENNAE[a]

Sensillum	Reaction to	%	Reaction to gland extract
Type A	*Z*5–10 : Ac (large spike amplitude) *Z*5–10 : OH (small spike amplitude) *Z*8–12 : Ac (small spike amplitude)	68	Activated
Type B	*Z*7–12 : Ac (large spike amplitude)	28	Activated
Type C	*Z*9–14 : Ac (large spike amplitude)	2	Activated
Type D	"Silent type" (no reaction)	2	
		100	

[a] All compounds listed in Figure 4 except ES–10 : Ac were tested. In addition, every sensillum type was tested for activation by a female abdominal tip extract (25 FE).

with large spike amplitudes; the following two components activated cells with small spike amplitudes. The omission of *E*5–10:Ac from the 100 sensilla trichodea series was rectified afterwards to some extent by testing a limited number of the A and B type sensilla (Table 2). However, *E*5–10:Ac did not elicit any single-cell activity from these sensilla.

Three different cells identical to the earlier-mentioned *Z*5–10:Ac, *Z*7–12:Ac, and *Z*9–14:Ac receptor were activated by the female extract.

Tube Olfactometry. Hexane, female glands, and a selection of 12 synthetic stimuli were tested for behavioral activity. The selection of synthetic compounds to be tested was based on the chemical analyses and electrophysiology (Figure 4, Table 2).

Neither *Z*5–10:Ac nor *Z*7–12:Ac applied in dilution series elicited a significant behavioral response, with the exception of wing-fanning (Table 3). The lowest amount tested approximately corresponded to the content of that specific compound in one excised female gland. The concentration was subsequently increased in decadic steps.

Contrary to single substances, combinations of compounds in proportions similar to those found in female glands gave rise to significant extension of genital claspers and copulation efforts. The activity of a four-component blend consisting of *Z* 5–10 : Ac, 10 : Ac, *Z* 7–12 : Ac, and *Z* 9–14 : Ac was as high as that of a female gland (Table 3).

Field Attractancy Test. The most active laboratory stimulus was compared with the attractivity of virgin females in the field. Traps baited with 10:Ac (1.25 μg), *Z*5–10:Ac (1.0 μg), *Z*7–12:Ac (12 μg), and *Z*9–14:Ac (10 μg) caught significantly more moths than traps baited with living females ($P < 0.01$, Wilcoxon matched-pairs signed-ranks test, two-tailed). The average catch per trap and night was 3.5 and 1.3 males, respectively. The

TABLE 3. NUMBER OF MALE *A. segetum* RESPONDING TO DIFFERENT STIMULI IN A TUBE OLFACTOMETER (N = 10)[a]

Stimulus and amount (ng)	Wing-fanning	Orientation	Extension of genital claspers	"Copulation"
Hexane	4 a	4 abc	0 a	0 a
*Z*5-10 : Ac				
0.1	5 a	2 a	1 ab	1 ab
1	9 a	5 abc	0 a	0 a
10	6 a	7 bc	0 a	0 a
100	7 a	5 abc	0 a	0 a
*Z*7-12 : Ac				
1	9 a	6 abc	0 a	0 a
10	8 a	4 abc	0 a	0 a
100	7 a	3 abc	0 a	0 a
*Z*5-10 : Ac (0.02) + *Z*7-12 : Ac (1)	5 a	3 abc	1 ab	0 a
*Z*5-10 : Ac (0.02) + *Z*7-12 : Ac (1) + 10 : Ac (0.04)	4 a	4 abc	2 ab	1 ab
*Z*5-10 : Ac (0.02) + *Z*7-12 : Ac (1) + *Z*9-14 : Ac (1)	8 a	1 a	0 a	0 a
*Z*7-12 : Ac (1) + *Z*9-14 : Ac (1) + 10 : Ac (0.04)	9 a	2 ac	0 a	1 ab
*Z*5-10 : Ac (0.02) + *Z*7-12 : Ac (1) + *Z*9-14 : Ac (1) + 10 : Ac (0.04)	9 a	7 bc	6 b	4 b
Female gland	8 a	8 b	5 b	4 b

[a]Numbers in the same column followed by the same letter are not significantly different at the 5% level (chi-square test with Yates correction).

number of moths trapped which belonged to other species in relation to the number of *A. segetum* males trapped was 12.5% in the female-baited traps and 6.3% in the traps baited with synthetic pheromone.

DISCUSSION

In the present study 13 pheromone candidates were isolated from female gland extracts. Seven of these compounds could be characterized unequivocally by chemical means: 10:Ac, 12:Ac, 14:Ac, 16:Ac, Z7-12:Ac, Z9-12:Ac, and Z9-14:Ac. In addition, EAG and single-cell experiments firmly sup-

ported the presence of *Z*5–10:Ac. While *Z*- and *E*5–10:Ac could not be resolved by GC, only receptor cells specialized to *Z*5–10:Ac were found on the male antenna. The second tetradecenyl acetate found (compound 9, Figure 2, Table 1) was present in small amounts. No specialized receptors were found on the male antenna for the five candidate isomers suggested by GC-MS. Therefore no further chemical characterization was attempted. The hexadecenyl acetate isomer was identified with certainty only in the final stage of our work, and no trials to determine the double-bond configuration were made.

The chemical analysis results are consistent with those of Tóth and coworkers (1980), who identified *Z*7–12:Ac and *Z*9–14:Ac, in the ratio 4:1, as the two major straight-chain monoolefinic acetates in *Agrotis segetum* female pheromone glands. Bestmann et al. (1978) reported *Z*5–10:Ac which is quantitatively a minor component in our animals.

On the other hand, there are both quantitative and qualitative discrepancies between the results obtained by Arn et al. (1980) and our results. Arn and coworkers reported *Z*9–12:Ac to be the most abundant dodecenyl acetate isomer, although of no certain behavioral function. In our study this isomer only amounts of 7% of the major compound, *Z* 7–12:Ac. Moreover, the 12:Ac is a minor compound compared to *Z*7–12:Ac. We could not detect *E*5–12:Ac and *Z*8–12:Ac, which were among the seven compounds found by Arn et al. The detection limit of our general analytical procedure is about 100 pg. On the other hand it is possible that small amounts of *Z*8–12:Ac eluted close to the major compound *Z*7–12:Ac on the columns used for this study and were therefore unresolved. We know of no well-documented cases, however, where the major pheromone-like compound in the gland extract is not also a primary sex pheromone component (Roelofs and Cardé, 1977). Such is the case for *A. segetum* where *Z*7–12:Ac was shown to be the major component.

Contrary to the work of Tóth et al. (1980), we have not shown *Z*9–14:Ac to be present in the female effluvium. It is possible that the number of females used for entrainment was too low. Decreasing yield for compounds of lower volatility was also a problem. Using a male *A. segetum* anenna for GC-EAD analysis of potential *Z*9–14:Ac-containing samples had no advantage compared to GC-FID because of the low antennal sensitivity to that compound.

Specialized receptors on the male antenna were found for only five of the 20 compounds listed in Figure 4. Three of these receptor cells reacted with large spike amplitudes to *Z*5–10:Ac, *Z*7–12:Ac, and *Z*9–14:Ac, respectively, and they were all activated by the female extract. In tube olfactometry, a combination of these three compounds with the addition of 10:Ac, at low concentrations, elicited strong close-range behavioral responses such as extension of genital claspers and copulation efforts towards the stimulus applicator. Thus we conclude that a mixture of 10:Ac, *Z* 5–10:Ac, *Z* 7–12:Ac, and *Z* 9–14:Ac in a ratio similar to that found in calling females is a good

approximation of the pheromone of *A. segetum*. Although no specialized receptors were found in single-cell screening, 10:Ac seemed to be essential for full behavioral activity in the laboratory behavioral bioassay. In the field, a combination of the compounds in the same ratio (with the absolute amounts based upon 1 μg of *Z*5–10:Ac) was three times more attractive than virgin females. This blend should be compared with that recommended by Arn et al. (1980). The activity of their blend (also based upon 1 μg *Z*5–10:Ac) was reported as being the same as of virgin females.

The ratio of substances in our synthetic blend used in the field experiments was based on a preliminary GC-MS quantification in an early stage of our work. Compared to the final GC-MS figures reported in this study, the proportion of *Z*9–14:Ac in the lures was probably too high. For *Z*7–12:Ac and *Z*5–10:Ac Arn et al. (1980) reported that a ratio higher than 2:1 was inhibitory. We have used a ratio of 12:1 in field experiments and still obtained very high activity. Unpublished field experiments (Löfqvist and Löfstedt, in progress) indicate that the optimal ratio between *Z*7–12:Ac and *Z*5–10:Ac is higher than 2:1 but lower than 12:1. However, different geographical races might produce and respond to different pheromone blends.

In *A. segetum* we could not find *Z*7–10:Ac, which has been reported to constitute 47% of the amount of the major pheromone component *Z*5–10:Ac in *A. fucosa* (Wakamura, 1978, 1980). Neither a significant EAG activity nor specialized receptors were revealed when male *A. segetum* antennae were examined. This suggests that the two species, although very similar according to Steck et al. (1979), may be reproductively isolated under natural conditions. It is possible that the gene flow between the two populations is limited by the differences in the composition of their pheromone. In this case one should reconsider whether *A. fucosa* and *A. segetum* are synonymous.

Sex pheromones or sexual attractants have been reported for six species belonging to the genus *Agrotis* (*Scotia*) (Table 4). All components described are acetates with 10-, 12-, or 14-carbon atom chains in their alcohol moiety. Moreover, with the probable exception of *Agrotis segetum* pheromone components, all reported compounds are monounsaturated. The present paper strongly indicates that the pheromones of *A. segetum* is a combination of one saturated and several monounsaturated compounds. Other examples of such a combination are *Argyrotaenia velutinana* (Roelofs et al., 1975), *Adoxophyes* sp. (Tamaki, 1979), and *Archips argyrospilus* (Roelofs et al., 1974).

The pheromone of *A. segetum* proposed in this study has been successfully used in a monitoring program for the turnip moth during the summer of 1981.

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TABLE 4. COMPARISON OF SEX PHEROMONE AND SEX ATTRACTANT COMPONENTS IN MOTHS BELONGING TO THE GENUS *Agrotis*

Species	10 : Ac	*Z*5–10 : Ac	*Z*7–10 : Ac	*Z*5–12 : Ac	*Z*7–12 : Ac	*Z*5–14 : Ac	*Z*9–14 : Ac	Reference
A. segetum[a]	X[c]	X	O	O	X	?	X	This study
A. fucosa[a]	–	X	X	–	–	–	–	Wakamura, 1978, 1980
A. ipsilon[a]	–	–	–	–	X	–	X	Hill et al., 1979
A. orthogonia[b]	–	–	–	X	X	–	–	Strubel and Swailes, 1978
A. venerabilis[b]	–	X	–	–	X	–	–	Steck et al., 1979
A. exclamationis[a]	–	–	–	–	–	X	X	Bestmann et al., 1980

[a] Compounds isolated from females.
[b] Compounds identified by field screening.
[c] X = compound present, O = compound not found, – = not reported, ? = compound might be present.

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Continuous single sensillum recording as a detection method for moth pheromone components in the effluent of a gas chromatograph

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ABSTRACT. Single sensillum recordings were made from male antennal pheromone receptors of *Agrotis segetum* (Schiff.) (Noctuidae) and *Adoxophyes orana* (F.v.R.) (Tortricidae). A gas chromatograph (GC) was used to prepare and to deliver the odour stimuli. Samples of female abdominal gland extracts were injected into the column of the GC and the responses of the receptor cells to the effluent were recorded continuously. The receptor cells responded with an increase of their action potential frequency during elution of the major pheromone components. The pheromone receptors of *Agrotis* showed a much higher sensitivity than those of *Adoxophyes*. In the extract of female glands from *Agrotis*, compounds were detected which have not been identified in previous studies of the sex pheromone of this species. It is suggested that the combination of GC techniques with direct single sensillum recording may serve as a valuable supplement to electro-antennographic techniques.

Key words. *Agrotis segetum, Adoxophyes orana,* Lepidoptera, gas chromatography, single-sensillum recording, pheromone detection, antennae.

Introduction

Gas chromatography (GC) is an indispensable technique in the detection and identification of insect pheromone components. It is limited, however, by the sensitivity of the detector and the resolving power of the column. Moreover, conventional detectors are not selectively sensitive to biologically active compounds. The olfactory receptors on insect antennae, on the other hand, show high sensitivity and high selectivity towards conspecific sex pheromones. The electroantennogram (EAG) technique (Schneider & Hecker, 1956; Schneider, 1957a, b) has therefore been applied to monitor such compounds in fractions collected from the GC effluent (Roelofs *et al.*, 1971; Persoons *et al.*, 1974; Bestmann *et al.*, 1980; Conner *et al.*, 1980).

Collecting fractions for subsequent EAG test is, however, laborious and may result in loss of both material and resolution. Moorhouse *et al.* (1969) used a glass collector permanently installed in the gas chromatograph and flushed the contents of the collector every 15 s over the electro-antennographic detector. Arn *et al.* (1975) equipped the gas chromatograph with an EAG detector which continuously monitored the effluent of the column.

This GC-EAG combination has been used as a selective and sensitive tool in the gas chromatographic analysis of insect pheromones, but use of single cell recording (Kaissling, 1971), which offers a higher selectivity than EAG recording, would be a logical refinement.

Different pheromone components have

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been shown to activate different receptor cells in the same olfactory sensillum. The responses of these cells can usually be discriminated by the differences in the amplitudes of their action potentials (O'Connell, 1972, 1975; Kaissling, 1974; Kochansky *et al.*, 1975; Den Otter, 1977; Van Der Pers, 1982).

In 1981 we tested GC fractions of a female pheromone gland extract of *Dioryctria abietella*. One of the fractions, collected in cooled pasteur pipettes, elicited single cell responses in conspecific male antennal sensilla. This response indicated that a direct recording of the GC effluent would be possible. We therefore combined the gas chromatograph with a set-up for single cell recordings. Two moth species, whose pheromones were already known, *Agrotis segetum* (Arn *et al.*, 1980; Löfstedt *et al.*, 1982) and *Adoxophyes orana* (Tamaki *et al.*, 1971), were used to test the method. Morphological characteristics of the antennal olfactory sensilla of these species have been described previously (Hallberg, 1981; Den Otter *et al.*, 1978).

Materials and Methods

Agrotis segetum (Schiff.) (Noctuidae) moths were obtained from the culture used by Löfstedt *et al.* (1982). *Adoxophyes orana* (F.v.R.) (Tortricidae) pupae were obtained from a culture at the Department of Zoology, Groningen University, Haren (The Netherlands). The insects were sexed as pupae. After emergence they were kept in plastic jars and provided with 5% sugar solution. Experimental animals ranged in age from 2 to 5 days post emergence.

Various concentrations of pure synthetic pheromone components were prepared in hexane. Extracts of female pheromone glands of both species were prepared as described by Löfstedt *et al.* (1982) at the time of maximum calling.

GC separations of gland extracts were performed on a Hewlett Packard 5835 gas chromatograph. Samples (1–3 μl) were injected onto a WCOT fused silica capillary column (20 m x 0.19 mm i.d.) coated with AT 1000 (Alltech, Inc.). The linear nitrogen flow at 60°C was 13 cm/s. The injector temperature was 250°C. Column temperature was maintained at 60°C for 5 min following injection, and then heated to 200°C at a rate of 15°/min or 20°/min.

An adjustable micro needle-valve effluent split (Scientific Glass Engineering, Inc.) was inserted between the column and the flame ionization detector to obtain an additional outlet for the single sensillum detector system. The split ratio between the ionization and sensillum detectors was about 2 : 1.

The effluent from the outlet for the sensillum detector was mixed with an airstream (charcoal filtered and humidified), flowing at 0.5 m/s through a glass tube (15 cm long, 7 mm i.d.). The downstream end of the tube was 15 mm away from and directed towards the antennal preparation. Experiments were conducted at room temperature, at about 20°C.

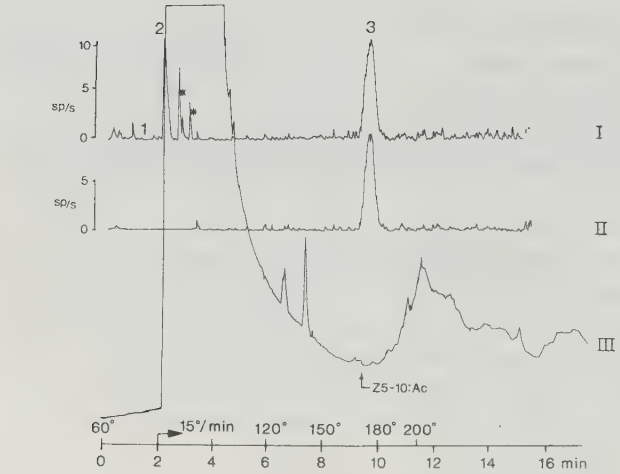
Recordings were made from single antennal sensilla trichodea on excised antennae of males. The action potentials generated by the receptor cells associated with these sensilla were recorded by the tip recording technique (Kaissling, 1974) as modified by Van Der Pers & Den Otter (1978). Before each experiment the sensilla were tested for their responsiveness by application of an air puff containing a synthetic test stimulus. The signals were continuously recorded on an instrumentation tape recorder and monitored on an oscilloscope. A 10-Hz time signal was recorded simultaneously.

After each experiment, recordings were transferred to paper using an ink jet recorder. Plots of action potential frequency against time were made separately on a paper recorder by detecting the action potentials with a level discriminator followed by a frequency-to-voltage converter (Murphy Developments, Haren, The Netherlands).

Results

After contact was achieved with a sensillum trichodeum type SW1 (Hallberg, 1981) on the antenna of a male *A.segetum*, a low spontaneous discharge of three receptor cells was recorded. The activities of these cells could be discriminated by the amplitudes of their action potentials.

In the first experiment, the spontaneous



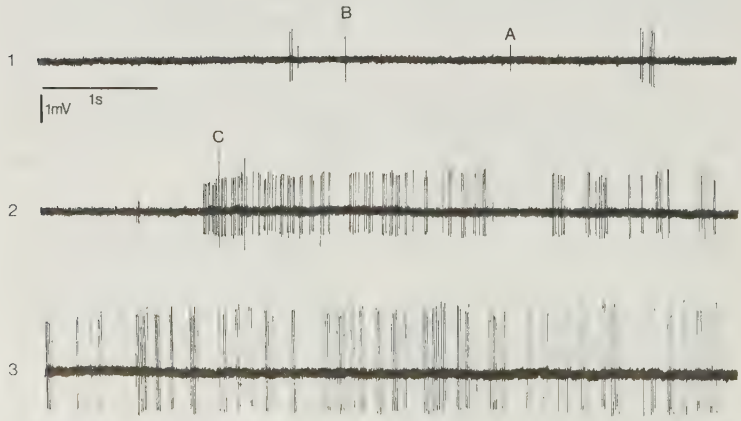


FIG. 1. Typical spike frequency responses from a single sensillum recording from a male *Agrotis segetum* antenna (traces I and II) stimulated with GC (gas chromatograph) effluent following injection into the GC of half a female equivalent extract from a female *A.segetum* sex pheromone gland; the contemporary flame ionization detector output is shown in trace III. Trace I, activity of cells B and C together; trace II, same for cell C alone. Ordinate, elapsed time from sample injection (at 0) and GC column temperature. The peaks asterisked in trace I due to 50-Hz line noise. Lower figures: details of upper traces at points indicated as 1, 2 and 3. A, cell A; B, cell B; C, cell C. Time and voltage scale refer to traces 1, 2 and 3.

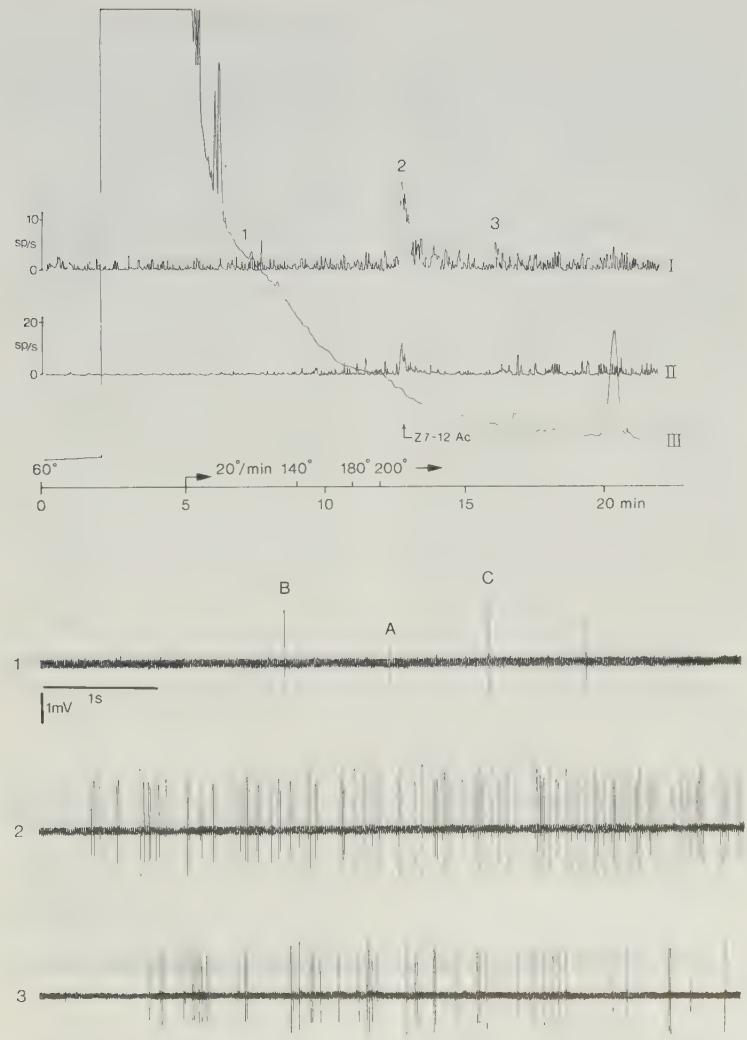


FIG. 2. Typical single sensillum responses obtained after injection into the GC of a female gland extract of *Agrotis segetum*. Separate antenna and recording from that in Fig. 1; all details otherwise the same.

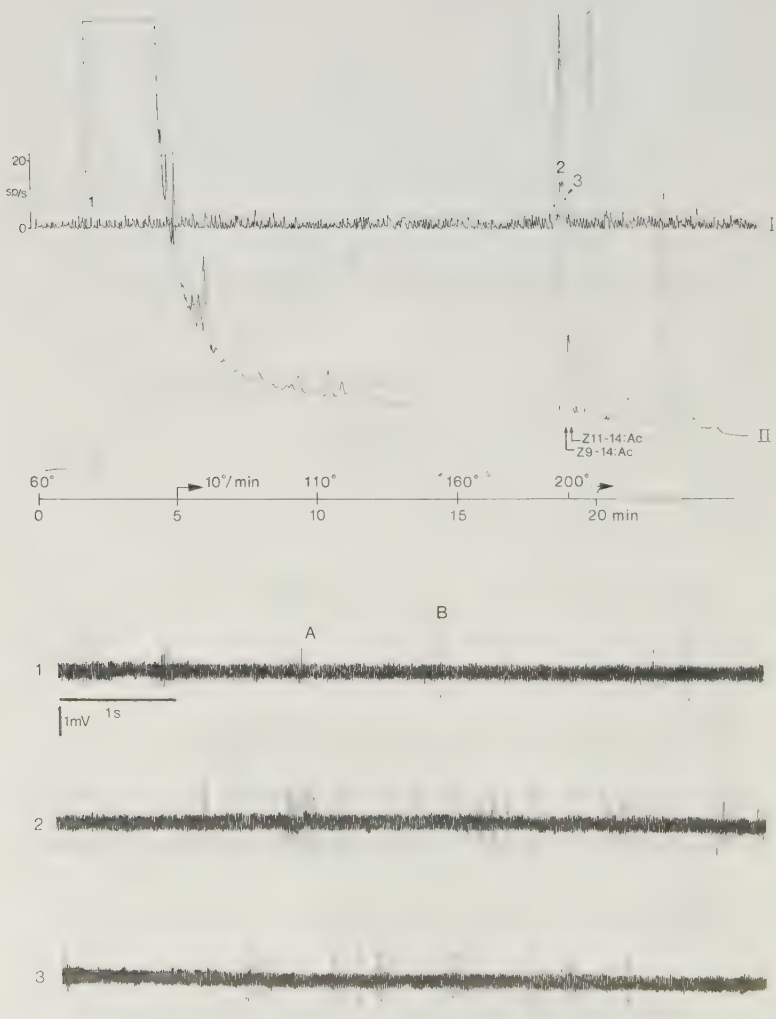


FIG. 3. Typical responses obtained from single sensillum recording of a male *Adoxophyes orana* antenna after injection into the GC of a female gland extract of *A.orana*. Trace I, activity of cells A and B; other details as for Fig. 1.

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discharge frequency (calculated from a 60-s recording) of the cells was: cell A (small spike amplitude) 0.48 spikes/s; cell B (medium spike amplitude) 0.45 spikes/s; cell C (large spike amplitude) less than 1 spike/min. An air puff containing (*Z*)-5-decenyl acetate (*Z*5–10:Ac) blown directly over the antenna highly activated cell C.

The responses when, after this, a 2-µl sample containing half a female equivalent of an abdominal tip extract in hexane was injected into the gas chromatograph are shown in Fig. 1. In this example, an instantaneous increase in the spike frequency of cell B was recorded 1 min 59 s after injection of the sample (trace 2 in Fig. 1). The response lasted for 8 s, and was followed by a gradual return to the level of spontaneous firing frequency over about 6 s.

A high response of cell C started 9 min 7 s after injection of the sample, reaching a maximum (11 spikes/s) at 9 min 24 s after injection lasting about 45 s (trace 3 in Fig. 1). The discharge pattern of this cell consisted of short bursts of three to eight spikes with small interspike intervals. During this response no significant response of the flame ionization detector could be recorded (cf. trace III in Fig. 1 at time of peak in trace II). No other responses from any of the three cells were recorded during this experiment. Following this experiment, which was terminated after 15 min, the retention time of a sample (1 ng in 1 µl hexane) of synthetic *Z*5–10:Ac was determined under the same conditions; maximum elution occurred at 9 min 19 s.

A second experiment was performed using another male antenna of *A.segetum*, contact again being made with a SW1 type sensillum. This time an air puff containing (*Z*)-7-dodecenyl acetate (*Z*7–12:Ac) activated cells in the sensillum, whereas an air puff containing *Z*5–10:Ac did not. During the experiment a low spontaneous activity of three cells was recorded. The cells were designated A (small spike amplitude), B (medium spike amplitude) and C (large spike amplitude) cells, and their spontaneous firing rates were 0.1, 1.1 and 1.0 spikes/s, respectively.

Cells B and C showed an increase in spike frequency at 12 min 44 s after injection of a female gland extract. Maximum responses for cell B (13.4 spikes/s) and cell C (11.3 spikes/s) were recorded at about 12 min 45 s after injection (trace 2 in Fig. 2). A second response of both cells was observed at about 16 min after injection (trace 3 in Fig. 2). During activation of these cells no significant response in the flame ionization detector was recorded (cf. trace III in Fig. 2).

This experiment was repeated using the same antenna but another SW1 sensillum also containing cells sensitive to *Z*7–12:Ac. In this case only a response from cell B was recorded, at 12 min 45 s after injection, whereas the activity of cell C did not change significantly. Again no flame ionization detector response associated with the activation of this cell was recorded. The retention time of synthetic *Z*7–12:Ac was established as 12 min 50 s under the same conditions.

Similar recordings from a sensillum trichodeum (Den Otter, 1977) on a male antenna of *Adoxophyes orana* showed spontaneous activity of two cells, designated as A (small spike amplitude) and B (large spike amplitude) with a spontaneous activity of 0.8–2 and 0.1–0.8 spikes/s, respectively. An air puff containing (*Z*)-9-tetradecenyl acetate (*Z*9–14:Ac) activated cell B. The discharge frequency of this cell increased at 18 min 35 s after injection of an *A.orana* female gland extract into the GC (Fig. 3, trace I). About 10 s later a slight increase of the firing frequency of the A cell was observed (traces 2 and 3, Fig. 3). These activities corresponded with a large response of the flame ionization detector (trace II, Fig. 3). The retention times of synthetic *Z*9–14:Ac and *Z*11–14:Ac were established 18 min 44 s and 18 min 54 s, respectively. The receptor cells did not respond to the other peaks in the flame ionization response appearing in trace II (Fig. 3).

Discussion

The sex pheromone of *A.segetum* has been reported to consist of a mixture of several components (Bestmann *et al.*, 1978; Tóth *et al.*, 1980; Arn *et al.*, 1980; Löfstedt *et al.*, 1982). The literature is not consistent about the definite composition of the pheromone blend, but the presence of *Z*5–10:Ac and *Z*7–12:Ac seems to be undisputed. Electro-

antennogram recordings using synthetic *Z*5–10:Ac and *Z*7–12:Ac revealed a high activation of olfactory receptors on the male antenna of *A.segetum* (Löfstedt *et al.*, 1982). The receptor cells sensitive to these compounds have been found to be present in electrophysiologically different SW1 types of sensilla trichodea (Löfstedt *et al.*, 1982; Hallberg, 1981). These sensilla contain two or three receptor cells (Hallberg, 1981).

In our experiments, activity of three receptor cell types (as characterized by the amplitudes of their action potentials) was recorded from SW1 sensilla trichodea. In accordance with the results of Löfstedt *et al.* (1982) the cells responding to *Z*5–10:Ac and *Z*7–12:Ac were found in different sensilla, although these sensilla were morphologically indistinguishable. The same cells responded to the synthetic compounds as well as to these compounds present in the female gland extracts. Only one cell (cell C, Fig. 1) was activated by *Z*5–10:Ac. One or two cells (cells B and C, Fig. 2) were activated by *Z*7–12:Ac.

The two experiments on a sensillum containing cells sensitive to this compound thus demonstrate that the number of cells sensitive to *Z*7–12:Ac is different in SW1 sensilla trichodea.

The sensitivity of the receptor cells appears to be high. The amounts of *Z*5–10:Ac and *Z*7–12:Ac isolated from an extract of one female gland are on the average about 60 and 1000 pg, respectively (Löfstedt *et al.*, 1982). This implies that in our experiments using injection of 0.5 female equivalents of a gland extract and with 33% of the GC effluent passed over the antenna (see Methods), the amounts actually reaching the antennal preparation were about 10 and 170 pg, respectively. 10 pg is well below the detection threshold of the flame ionization detector. The antennal receptor cells, however, were clearly activated by these low amounts (Figs. 1 and 2).

Löfstedt *et al.* (1982) reported that the sensillum containing the cell responding to *Z*5–10:Ac also contains a cell sensitive to *Z*-5-decenol (*Z*5–10:OH) and *Z*8 12:Ac. This cell is characterized by a medium spike amplitude. We did not record any change in activity of such a cell which could be attributed to the presence of these compounds in the female gland extract, however. If present, the concentration of these compounds in the extract must have been too low to activate the receptor cells. The amount of *Z*5–10:OH found by Löfstedt *et al.* (1982) appears to be half the amount of *Z*5–10:Ac, whereas they could not detect any trace of *Z*8–12:Ac in the extracts.

Specialized receptor cells may occur in relatively low numbers. Löfstedt *et al.* (1982) identified *Z*9–14:Ac in female gland extracts in amounts of about 500 pg/female. They recorded from a male antennal sensillum containing a cell highly sensitive to this compound, but the cell occurred in only two out of the 100 sensilla tested and was never associated with cells sensitive to *Z*5–10:Ac or *Z*7–12:Ac.

This may explain why we did not find activation of receptor cells at the retention time of *Z*9–14:Ac. On the other hand, we did record activity which cannot be attributed to any of the pheromone components identified so far. The early response of the B cell in the first experiment (Fig. 1) is probably not evoked by a pheromone-like substance but rather by a compound with a much higher volatility. Judging from the GC retention time, the response of the B and C cells in the second experiment (Fig. 2) may be due to a compound with a relatively low volatility. These compounds, however, have not yet been identified.

Our experiments show that the olfactory communication system in *A.segetum* may be complicated. Not only the number of components in the female sex pheromone (Arn *et al.*, 1980; Löfstedt *et al.*, 1982) but also the number of different receptor cells appear to be high. Moreover, these cells occur in different types of sensilla trichodea with similar morphological appearances. Comparable results have been found for the pheromone receptors in several species of *Yponomeuta* (Van Der Pers, 1982).

Single sensillum response to female sex pheromone components in male *Adoxophyes orana* have been analysed extensively by Den Otter (1977). The male sensilla trichodea contain three cells. One cell responds mainly to *Z*11–14:Ac; another mainly to *Z*9–14:Ac. Both compounds have been identified as

present in the female sex pheromone (Tamaki *et al.*, 1971). The function of a third cell is still unknown.

In our *Adoxophyes* experiments, we recorded activity from two cells. Occasionally large spikes occurred, evidently due to a discharge of the third cell. After injection of a female gland extract into the GC one of the receptor cells (cell B) increased its firing frequency at the time of the elution of *Z*9–14: Ac. A slight activation of cell A could be recorded a few seconds later. The latter response may be attributed to *Z*11–14: Ac, which eluted slightly after *Z*9–14: Ac.

The gas chromatographic conditions used did not allow complete separation of the two compounds. Relatively large amounts of both pheromone components are present in the extract, as can be concluded from the high flame ionization detector response (Fig. 3). Consequently, a high response of both A and B cells would be expected. Den Otter (1977) found, however, an inhibitory effect of the B cell on the response of the A cell during stimulation with mixtures of synthetic *Z*11–14: Ac and *Z*9–14: Ac. His experiments show that the responses of the A cell to *Z*11–14: Ac applied alone are higher than those to the same concentrations of this substance when mixed with *Z*9–14: Ac.

Behavioural experiments by Den Otter & Klijnstra (1980), and chemical analysis of female gland extracts by Guerin *et al.* (1982) strongly indicate the presence of additional minor components in the sex pheromone of *A.orana*. Evidence for the presence of these components cannot be found in the results of the present study, however. It is possible that the concentrations of these components in the female gland are too low to be detected by a single receptor cell, or that there is a relatively small number of cells which are specifically sensitive to these components on the male antenna.

Comparison of the results of our experiments with *Agrotis segetum* and *Adoxophyes orana* shows a large difference in sensitivity between the pheromone receptors of these species. Very small amounts of pheromone substances elicit high responses of the receptor cells in *A.segetum*, whereas the relatively large amounts present in the gland extract of *A.orana* elicit only weak responses in the receptors of *A.orana*. There may thus be a negative correlation between the amount of pheromone substance produced by the females and the sensitivity of the conspecific male pheromone receptors.

Our results demonstrate the feasibility of detecting pheromone components in female gland extracts by single sensillum recording directly in the effluent of a gas chromatograph. By comparison with gas chromatograph-electroantennogram (GC-EAG) detection, the method has the advantage of offering the possibility of differentiating between responses of different receptor cells. In one GC-single sensillum experiment, however, information can be obtained about only one type of sensillum, whereas GC-EAG detection experiments provide information from many types of olfactory cells.

For this reason the GC-single sensillum technique should be considered a valuable supplement to but not as a substitute for GC-EAG detection. On the other hand, GC-single sensillum recording may be very useful in the detection of biologically active compounds such as pheromones and plant volatiles in species with antennae which do not elicit good EAG responses but are well-suited for single sensillum recording. We are now improving and extending the technique for the study of olfactory communication in several insect species.

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BEHAVIOURAL RESPONSES OF MALE TURNIP MOTHS *AGROTIS SEGETUM*[1] TO SEX PHEROMONE IN A FLIGHT TUNNEL AND IN THE FIELD.[2]

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ABSTRACT

The response of individual male turnip moths Agrotis segetum was observed in a sustained flight tunnel to a mixture of decyl acetate, (Z)-5-decenyl acetate, (Z)-7-dodecenyl acetate and (Z)-9-tetradecenyl acetate in proportions similar to those found in gland extracts from virgin females (0.6:1:5:2.5). Lures containing 3-30 ug (Z)-5-decenyl acetate proved to be maximally attractive with approximately 60 % of the males completing all behavioural steps from activation to copulation efforts. A 300 µg dosage caused significant arrestment of upwind flight. Peak response to synthetics, however, was significantly lower than to female glands. Omitting decyl acetate from the blend did not affect the activity while omission of any of the three monounsaturated acetates caused a dramatic decrease in response. In the field maximum trap catches were achieved with 1-30 µg lures. The subtractive assay carried out in the field confirmed the neutrality of decyl acetate and the importance of the three monoenes. Adding 1 % of (Z)-8-dodecenyl acetate (earlier reported as an "inhibitor") to the 4-component mixture decreased the trap catch to about 50 % and increasing the amount of (Z)-8-dodecenyl acetate to 27 % decreased the activity further to about 10 %. (Z)-8-dodecenyl acetate also decreased the number of successful flights in the flight tunnel.

Key Words: Agrotis segetum, turnip moth, Lepidoptera, Noctuidae, sex pheromone, flight tunnel, decyl acetate, (Z)-5-decenyl acetate, (Z)-7-dodecenyl acetate, (Z)-9-tetradecenyl acetate, (Z)-8-dodecenyl acetate, field tests, attraction.

INTRODUCTION

Bestmann et al. (1978) reported (Z)-5-decenyl acetate (Z5-10:OAc) to be a sexual attractant of the turnip moth, Agrotis segetum Schiff.(Lepidoptera: Noctuidae), based on chemical analysis, electroantennographic (EAG) activity and preliminary olfactometer tests. Later, efforts by several groups (Arn et al. 1980, Toth et al. 1980, Löfstedt et al. 1982 and Arn et al. 1983) revealed a much more complex, multicomponent pheromone in the turnip moth. Löfstedt et al. (1982) identified 13 aliphatic acetates and alcohols from female gland extracts. The pheromone consists of at least the three homologous monounsaturated acetates; Z5-10:OAc, (Z)-7-dodecenyl acetate (Z7-12:OAc) and (Z)-9--tetradecenyl acetate (Z9-14:OAc). In field trapping experiments these three compounds synergized each other, with no or only slight attractivity as single components or binary mixtures (Arn et al. 1983). Tube olfactometer experiments indicated the possible activity of decyl acetate (10:OAc) (Löfstedt et al. 1982), also present in the female pheromone gland.

Arn et al. (1980) also reported (Z)-8-dodecenyl acetate (Z8-12:OAc) as a predominant constituent of female A.segetum pheromone gland extracts, but assigned it an "inhibitory" function. When male A.segetum sensilla trichodea were screened (Löfstedt et al 1982) a receptor cell specialized to Z8-12:OAc and (Z)-5-decenol (Z5-10:OH) was found. We hypothesized that both Z8-12:OAc

and Z5-10:OH might have an "inhibitory" effect on A.segetum males by activation of a common receptor.

The present study comprises experiments, in a flight tunnel and in the field, designed to elucidate the behavioral activity of the above mentioned pheromone components and inhibitors.

MATERIALS AND METHODS

Insects. Turnip moths were maintained in the laboratory on a modified Hinks & Byers (1976) diet, using potatoes instead of pea beans. The sexes were separated as pupae and the males were kept in a separate room at 22° C, 40 % r.h. on a reversed 16 h light: 8 h dark photoperiod. Each male was kept in a 250 ml plastic jar and fed a 5 % sucrose solution. The males tested in the flight tunnel were the 10-15th generation offspring of insects collected in the field (Löfstedt et al. in prep.).

Chemicals. Unsaturated compounds used for the formulation of the lures were at least 98 % pure regarding geometrical and positional isomers. 10:OAc was purchased from Schuchardt, München, FRG, and (Z)-6-dodecenyl acetate (Z6-12:OAc), Z7-12:OAc, Z8-12:OAc, Z9-14:OAc and Z5-10:OH from the Institute For Pesticide research, Wageningen, The Netherlands. Z5-10:OAc was a gift from Bernt Thelin, Dept. of Organic Chemistry 3, Lund University. The rest of the compounds were from the laboratory collection of pheromone components from various sources.

Stock solutions of the single compounds were made up in heptane (1-10 µg/ul). Blends of components to be tested were made by mixing various amounts of the stock solutions and adding hexane to the desired concentration. The mixtures in 25-50 µl solvent were applied to rubbersepta (red 5x9 mm, Arthur H. Thomas, Co. U.S.A.) or polyethylene capsules (1 ml, 1.5 mm wall thickness, Kartell, Italy). The solvent was evaporated at room temperature, the capsules were closed, and stored at -20° C when not in use. Dosages are designated on their content of Z5-10:OAc. For the flight tunnel experiments with different ratios between the monoenes, fresh baits were made up daily, 4-5 h before the experiments.

The blend ratio in the 1982 experiments (10:OAc/Z5-10:OAc/ Z7-12:OAc/ Z9-14:OAc = 1.25:1:12:10) was based on the amounts isolated from female pheromone glands reported in Löfstedt et al. (1982). Later, more precise quantitative analyses of glands revealed a higher proportion of the volatile components (Löfstedt and Odham, 1984 (i.e. 10:OAc/Z5-10:OAc/Z7-12:OAc/Z9-14:OAc = 0.6:1:5:2.5). All experiments during 1983 were based on these new ratios.

Flight tunnel experiments. Experiments were performed in a 2.0 m long x

0.9 m diameter plexiglas flight tunnel that had a closed design, with the entire air volume exhausted after a single passage through the wind tunnel. Turbulence in the flow was reduced by stainless steel screens at the outlet as well as at the inlet end of the tunnel. A minor distortion of the flow was observed when the doors at the side of the wind tunnel were opened to introduce baits or moths. The experiment with different ratios between the homologous acetates was performed in an open windtunnel, with only the contaminated air ventilated out of the room. This tunnel had a square cross-section 0.9 x 0.9 m^2 . Both tunnels had a black and white striped floor.

Flight tunnel conditions were 20-21^{o}C, 35-40 % r.h. and 0.25 m/s wind speed. The light intensity was approximately 1 lux. In the majority of the experiments rubber septa to be tested in the flight tunnel were fixed at one end of a pipe cleaner, ca 15 cm long, which was attached to the screen at the upwind end of the tunnel. In this way the septum hung about 30 cm above the floor and at least 10 cm from the upwind end of the flight tunnel. Female glands were tested by placing 10 snipped glands on the head of an insect pin and securing the pin to the end of a pipe cleaner. The glands were prepared just prior to testing them in the tunnel. In the experiment with different ratios the septa were placed in a holder at the top of a 35 cm high steel-rod. Two to three day old male moths to be tested were transferred individually to 250 ml clear plastic cups and placed in the flight tunnel room prior to initiation of the dark period. In this way moths were allowed to acclimate to the low light intensity in the tunnel for at least three hours before they were tested 3-5 hours into the scotophase.

For the test a male moth was transferred to a cylindrical screen cage open at one end. Release cages were placed into the tunnel with the open end facing upwind, on a wire screen platform approximatly 25 cm above the tunnel floor, 1.5 m from the source. Males were allowed two minutes to respond and were scored for the following behaviours: taking flight, stationary orientation flight near the release cage, upwind anemotactic flight and source contact (see Linn and Roelofs 1981). Behaviours were recorded on a cassette tape recorder.

Field trapping experiments. Field tests were conducted in agricultural crops (carrots, lettuce, potatoes, beet roots or sugar-beets) outside Lund, southern Sweden.

The 1982 experiments were carried out between June 6th and June 24th using sticky wing traps (Albany Inc., Needham Heights, Mass., U.S.A.). In 1983 the field experiments lasted from June 30th to July 12th, and a trap similar to the Albany trap but constructed in our laboratory (Löfqvist and Jönsson, in preparation) was used.

Traps within a replicate were spaced at least 20 m apart, in a line at

right angle to the predominant wind direction. Replicates were separated by at least 150 m, and usually as much as 300 - 400 m.

Traps within a replicate were rerandomized as soon as the most attractive trap had caught 5-10 males. In this way positional effects were minimized. The total catch in each trap over the whole experimental period was generally treated as one replicate. Occasionally replicates in time were generated by dividing a trapping period into two. Trapped moths were counted and removed daily or every second day. The trap bottoms were replaced when they were about to lose their stickiness. The baits were renewed at least every week because of the short half-life of the 10-carbon compounds.

RESULTS

Dose-response experiments. In 1982 a blend of 10:OAc, Z5-10:OAc, Z7-12:OAc, and Z9-14:OAc was tested in the field at dosages ranging from 0.001 to 10 µg (Fig. 1). With both polyethylene caps and rubber septa the highest response occurred with the 10 µg treatment. The 1983 experiment, using rubber septa, revealed a maximum trap catch over the range of 0.3 to 30 µg. (Fig. 1).

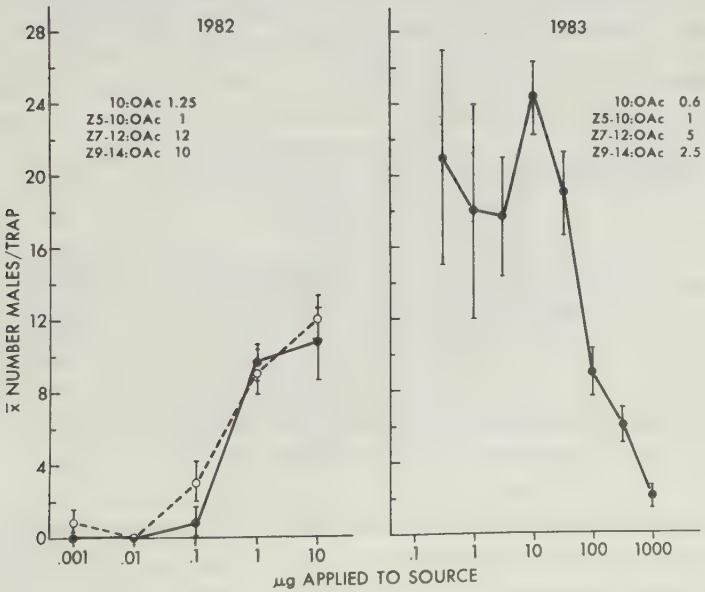


Figure 1: Mean number of A.segetum males (± SEM) attracted to different dosages of two mixtures of 10:OAc, Z5-10:OAc, Z7-12:OAc and Z9-14:OAc (n = 4 in both experiments). For 1982 the dashed line is for polyethylene caps, while the solid line for 1982 and 1983 is for rubber septa.

The 1983 ratio was also tested in the flight tunnel. Males responded in highest numbers to 3 and 30 µg treatments, with 54 and 59 % respectively of the males flying upwind and making contact with the source (Fig. 2). This response level was significantly lower, however, than that observed to female glands. Increasing the dose to 300 µg caused arrestment of upwind flight as well as a significant decrease in the number of males that initiated upwind movement, while decreasing the dose lowered the number of males orienting in the odour plume, with all of the males that successfully oriented also com-

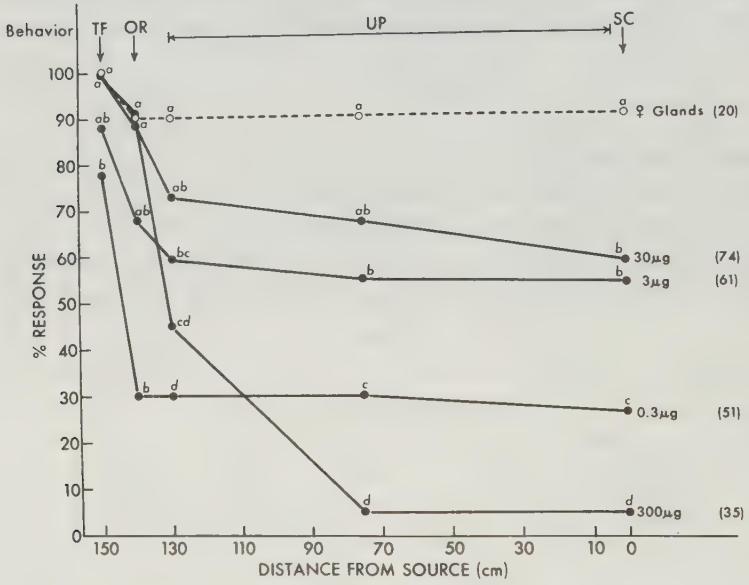


Figure 2: Percentage response of male A.segetum tested individually in the flight tunnel to female glands and four dosages of a synthetic four-component mixture of 10:OAc, Z5-10:OAc, Z7-12:OAc and Z9-14:OAc (0.6 : 1 : 5 : 2.5). The dose (µg) refers to the amount of Z5-10:OAc in the respective source. Behaviours are: taking flight (TF), orientation flight (OR), upwind flight (UP), and source contact (SC). Values in parentheses indicate the number of males tested to each treatment. Data points within each behaviour having different letters are significantly different, according to the method of adjusted significance levels for proportions ($p<0.05$).

pleting the upwind flight. Temporal analysis of the behavioural response showed that the time to proceed from the initial behaviour, activation (wing fanning), to any succeeding step in the flight sequence increased with increasing amounts of stimulus (Fig. 3), with the shortest duration for the behavioural sequence observed to female glands.

Tests with additional components. Several additional blends were tested during the 1982 field season (Fig. 4). The mixtures containing the unsaturated

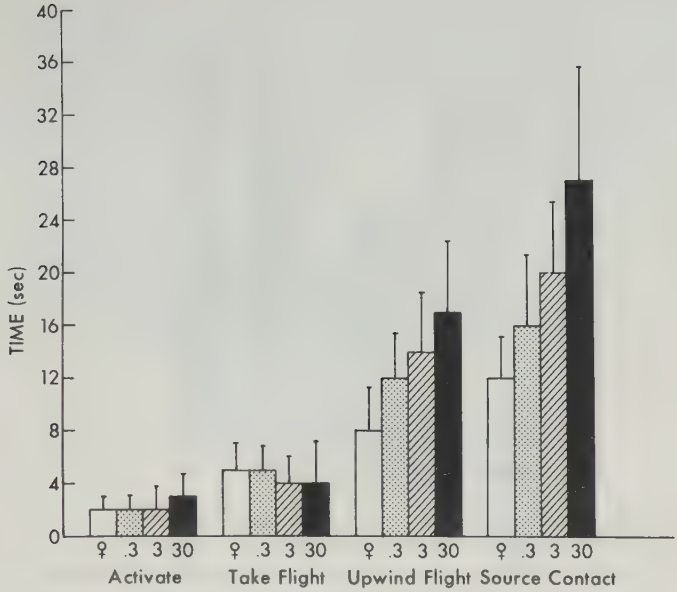
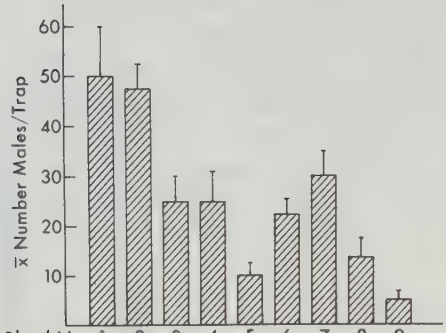


Figure 3: Time (seconds taken by male A. segetum to initiate four phases of the behavioural response to treatments in fig. 2 (300 µg dose excluded). Values are the means (± SEM) for the number of males completing the sequence to each treatment (see Fig. 2).

acetates Z5-10:OAc, Z7-12:OAc, and Z9-14:OAc in a 1:1:1 ratio were most attractive (TRT.1 and 2). As in the subtractive tests leaving out 10:OAc did not affect trap catches (TRT. 1 and TRT.3 compared to TRT.2 and TRT.4, respectively), while the omission of Z9-14:OAc did (TRT.5 compared to TRT.4). The relative activity appeared to be restored by the addition of a large amount of Z9-12:OAc (TRT. 6 and 7 relative to TRT. 4). The addition of Z5-10:OH (TRT. 8) or Z8-12:OAc (TRT. 9) reduced the trap catches. In 1983 a separate field experiment was designed to study the effect of the potential "inhibitor" Z8-12:OAc and a neutral compound, the isomer Z6-12:OAc, on trap catches. Whereas even small amounts of Z8-12:OAc reduced the trap catches, no such effect was observed for Z6-12:OAc (Fig. 5). When 150 µg of Z8-12:OAc (=the amount of the pheromone component Z7-12:OAc) was added to the 4-component mix at the 30 µg dose, the percentage of males taking flight decreased from 96 to 75%. The proportion of males reaching the source decreased from 60 to 0 % (N=25).

Subtractive assay. Removing any of the three unsaturated acetates from the 3 µg dosage of the 4-component blend tested in the 1983 field trials resulted in significantly fewer males being trapped (Fig. 6). In contrast the omission of 10:OAc from the blend did not significantly change the trap catch.

In flight tunnel tests with a 30 µg dosage of the 1983 4 component blend a pattern similar to that in the field was observed (Fig. 7). However, in the



Blend No.	1	2	3	4	5	6	7	8	9
10:OAc	–	8	–	1.25	1.25	–	1.25	1.25	1.25
Z5-10:OAc	8	8	1	1	1	1	1	1	1
Z7-12:OAc	8	8	12	12	12	12	12	12	12
Z9-14:OAc	8	8	10	10	–	–	–	10	10
12:OAc	–	–	–	–	–	100	–	–	–
E5-12:OAc	–	–	–	–	–	10	–	–	–
Z9-12:OAc	–	–	–	–	–	100	100	–	–
Z5-10:OH	–	–	–	–	–	–	–	10	–
Z8-12:OAc	–	–	–	–	–	–	–	–	10

Figure 4: Mean number of A.segetum males (± SEM) attracted to blends of pheromone candidates (n = 4). Values indicate the amount (µg) of each compound applied to rubber septum source.

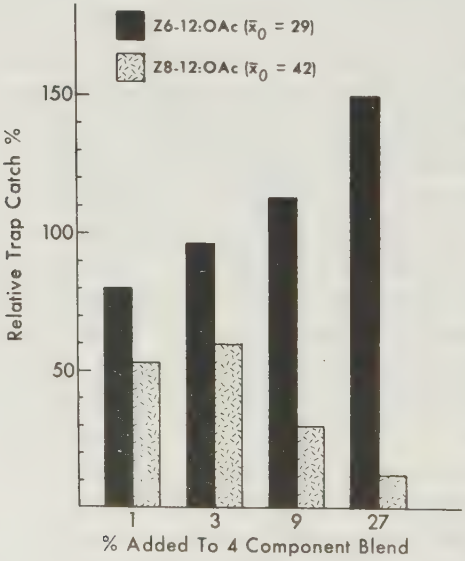


Figure 5: Mean trap catch (n = 4) relative to the four-component blend (10:OAc, Z5-10:OAc, Z7-12:OAc and Z9-14:OAc; 0.6 : 1 : 5 : 2.5) with two pheromone component analogues added. X_0 = mean trap catch with four-component control in the experiment with the respective analogue. The experiments were performed with the 3 µg dosage of the pheromone. Percent added is related to the total amount of the four-component blend, i.e. 27 µg.

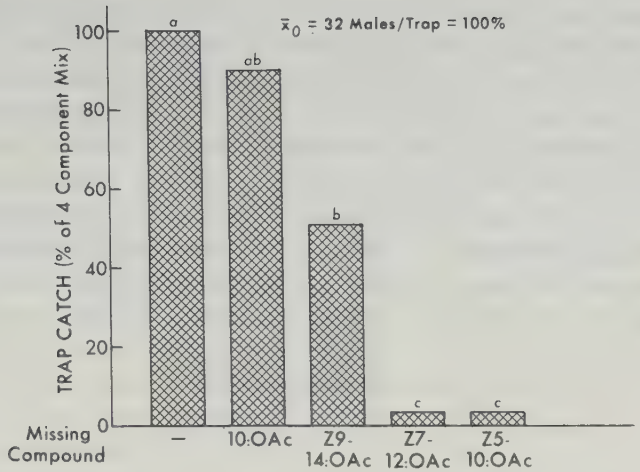


Figure 6: Mean trap catch (n = 4) relative to a four-component blend of 10:OAc, Z5-10:OAc, Z7-12:OAc and Z9-14:OAc (0.6 : 1 : 5 : 2.5) with one of the compounds subtracted. Significant differences are based on ANOVA ($\sqrt{X+0.5}$ transformations of the total catch) followed by comparison of means by Duncan's multiple range test. X_0 = average catch with four ocmponent blend.

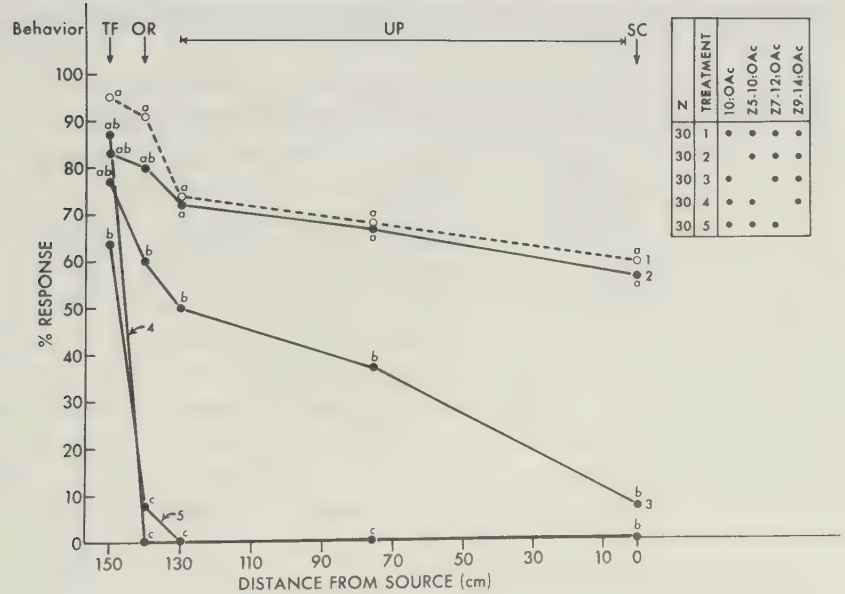


Figure 7: Percentage response of male A. segetum tested individually in the flight tunnel to 30 µg dosage of the four-component blend and the 4 three-component blends in Fig. 4. Behaviours and analysis as in Fig. 2.

flight tunnel removal of Z9-14:OAc (TRT.5) appeared to have a more dramatic effect on the behaviour than was observed in the field.

Comparison of two ratios of the homologous acetates. We compared the response of males to the 1:5:2.5 blend used by us, with the response to the 1:1:1 blend found to be optimal by Arn et al. (1983). At three different dosages (Table 1) there were no significant differences between the two blends. The maximal response was in both cases obtained with the 3 µg dosage.

At the 30 µg dosage many of the males initially orienting into the plume, never completed the upwind flight. Temporal analysis of the responses was not performed.

DISCUSSION

The optimal pheromone dose for attraction of A.segetum males in our experiments was within the 1 to 30 µg range, both in the field and in the flight tunnel. Field results using rubber septum and polyethylene capsule dispensers gave similar results, although half-lives of pheromone components on rubber septa (Butler & McDonough 1979) are much longer than on polyethylene capsules (Kuhr et al. 1972). In addition changes in component ratios between 1982 and 1983 did not seem to affect the optimal dose substantially. In both years maximum trap catch was achieved with dosages that correspond to 200-300 µg

Table 1. Responses of male Agrotis segetum in a flight tunnel to two blend-ratios of Z5-10:OAc/Z7-12:OAc/Z9-14:OAc at different dosages.

Dose	Ratio Z5-10:OAc/ Z7-12:OAc/ Z9-14:OAc	Response (%) a) Number tested	Taking flight	Stationary orientation	Source contact
0.3 µg	1:5:2.5	33	100	61a	30ab
	1:1:1	30	100	60a	23 b
3 µg	1:5:2.5	30	100	80a	47ab
	1:1:1	37	100	84a	57a
30 µg	1:5:2.5	30	100	77a	23b
	1:1:1	39	100	62a	23b

a) Values in each column followed by the same letter are not significantly different ($p \leq 0.05$) according to the method of adjusted significance levels for proportions (Ryan 1960).

total dispenser load. Arn et al. (1983), found maximum attraction to a 1:1:1 mixture of the monounsaturated acetates (10:OAc not included) with a 300 µg total septum load. Thus the optimal dose seems to be relatively independent of the blend ratios.

The flight tunnel experiments confirmed that as in earlier studies on the Oriental fruit moth Grapholita molesta (Baker & Roelofs 1981, Linn & Roelofs 1983) lower and upper behavioural thresholds (Roelofs 1978) are due to decreased activation and arrestment of upwind flight respectively. Temporal analyses of the male response added an important measure to the behavioural analysis. The increase in time necessary to proceed through the steps in the behavioural chain corresponds with the decreased ground velocity as an effect of increased pheromone concentration reported for flying moths (Cardé & Hagaman 1979, Kuenen & Baker 1983).

From the present study it is not possible to assign a specific function to any of the A.segetum pheromone components. Subtraction of any of the three monounsaturataed acetates affected all behavioural steps from activation to contact with the odour source, while 10:OAc seemed to be behaviourally neutral. Thus our study adds evidence to a more holistic perspective, with the total signal emitted affecting all steps in the behavioural response (Linn & Roelofs 1983, Linn et al. 1984). Flight tunnel and field data also correspond in this respect, except for the subtraction of Z9-14:OAc. Here the trap catch is higher than would be expected from the flight tunnel data. However, the approximately 50% reduction in trap catch is consistent with what Arn et al. (1983) found in their field test with the three unsaturated homologues in Denmark, close to southern Sweden. It is interesting that Z9-12:OAc seemed to substitute for Z9-14:OAc in the blend, when added in ten times higher amounts. This could be because of interaction with the Z9-14:OAc receptor (Löfstedt et al. 1982).

Our most active synthetic blend tested in the flight tunnel was inferior to pinned female glands. Although the difference was minor, studies with Trichoplusia ni (Linn et al. 1984) have shown that it should be possible to get a near 100% response with synthetic compounds in an assay of this kind. It should also be pointed out that the males responded faster to the glands than to any of the four-component dosages, and that the longest duration for the complete behavioural sequence was observed to the peak 30 ug synthetic blends. This can be compared to observations on G.molesta in which the duration of each phase of the behavioural sequence increased with the deviation from the optimal blend (Linn & Roelofs 1983). We think that an optimal stimulus is characterized not only by high numbers of responding individuals, but also by a rapid response.

In an attempt to rectify our use of what could have been a suboptimal

ratio between the monoenes, the 1:5:2.5 blend was compared with the 1:1:1 blend found to be optimal by Arn et al. (1983). A full exploration of different ratios and the possible impact of the pheromone dose on the optimal ratio (Linn and Roelofs 1983, Bellas and Bartell 1983) was not within the scope of this study. The experiment reported did not reveal any dramatic effect of the ratios on the number of males responding. None of the blends elicited completed up-wind flight in more than 50 % of the males. In this experiment males responding to the 30 ug dose of the 1:5:2.5 blend did not fly up-wind to the same extent as in the first dose-response experiment. A probable explanation for this is the use of fresh baits every day, resulting in relatively higher release rates.

We can see several explanations for the lower activity of synthetics in our assay. First, although the present study and other reports (Arn et al. 1983, Löfstedt et al. unpublished) have shown that the ratios between components is of minor importance, the disaccord in release rates from glands and rubbersepta might be enough to explain the difference. This can be tested by collection of airborne volatiles from dispensers and glands.

Second, pheromone components might be missing in the blend. Addition of Z9-12:OAc, 12:OAc and Z5-10:OH did not increase the activity significantly, but more aliphatic acetates and alcohols with possible pheromone activity are present in the gland (Löfstedt et al. 1982). Furthermore it is not unlikely that trace components in addition to these reported are present. Synergistic affects of less than 0.1% have been shown in Lepidoptera (Steck et al. 1982). Unfortunately the very low amounts of pheromone per female ($\leq$ 3 ng) makes analytical work with A. segetum demanding. Finally, it can not be ruled out that trace impurities in the compounds (<2 %) used to make up the synthetic blends could account for the reduced activity.

"Inhibition" of pheromone behaviour is a poorly understood area of research. Non-pheromone chemicals that negatively influence attraction, have been referred to as "inhibitors", "maskers" or "anti-attractants" in various contexts (Cardé 1976). Our study reveals the mode of action of Z8-12:OAc on male A.segetum pheromone behaviour as mainly arrestment of upwind flight. In contrast the Z6-12:OAc isomer did not have this negative effect but rather increased the attraction, while Z5-10:OH which activates the same receptor as Z8-12:OAc also reduced trap catch. Obviously a negative activity can not be assigned to every pheromone analogue. Arrestment of upwind flight evoked by activation of a specific receptor cell is probably too simple an explanation, but it remains a testable and non-falsified working hypothesis.

We can imagine at least two groups of negative compounds: ecologically meaningful compounds, important in intra- and/or inter-specific communication, and synthetic compounds not naturally occurring. The later group is of no eco-

logical relevance, but might still be of potential use in pest control. Z8-12:OAc is not a very common pheromone component in moths; it has only been found in a few tortricids (Roelofs and Brown 1983) and the adaptive significance of a male receptor-cell tuned to such a compound that is not used in the sex pheromone is unclear.

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Analysis of Moth Pheromone Acetates by Selected Ion Monitoring Using Electron Impact and Chemical Ionization Mass Spectrometry

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Mass spectrometric determination of moth pheromone components by means of selected ion monitoring with electron impact and chemical ionization has been evaluated. For the analysis of acetates, chemical ionization with ammonia as reactant gas proved advantageous with respect to sensitivity and selectivity. A quantitative assay for determination of pheromone samples from individual turnip moth females (*Agrotis segetum*, Noctuidae), using internal standards, deuterated at the acyl group, was developed. The assay showed correct linearity in the investigated region (20–2000 pg). Signal to noise ratios for 100 pg of acetates using chemical ionization (ammonia as reactant gas) were in the range 10–50 depending on the degree of unsaturation. Conservative limits of detection (background signal + three times the standard deviation) and quantification (background signal + 10 times the standard deviation) were approximately 70 and 240 pg, respectively. A significant decrease in gland titre of (*Z*)-5-decenyl acetate and (*Z*)-7-dodecenyl acetate after mating was established.

INTRODUCTION

Moth sexual attractants have been intensively investigated for more than 20 years since the first characterization of such a compound—the silkworm sex attractant.[1] However; qualitative and quantitative analyses of moth pheromones still constitute intricate chemical problems.

The moth sex attractants are generally produced in the female pheromone gland in nanogram or subnanogram quantities.[2] As a consequence, their characterizations have usually involved preparation of extracts of hundreds or even thousands of insects (see for example Ref. 2). Recent developments in isolation and of analytical techniques have, however, made it possible to analyse individuals for pheromone contents, but then only in certain species producing at least 10–20 ng of active components.[3,4]

In contrast to conventional analyses of homogenized mixed samples, the possibility to study pheromones on the individual level may provide information on the variances within populations. For example, examination of the chemical basis for sexual selection, the degree of chemical separation between different populations, and the genetics concerning pheromones may be performed.

Gas chromatography with flame ionization detection (FID) allows quantitative studies down to the low nanogram level, whereas the use of electron capture detection (ECD) may extend the work to the picogram region. However, the latter analyses of moth pheromones require sample derivatization.[5]

Mass spectrometry provides, particularly when combined with gas chromatography, an ideal means of identifying and quantifying pheromones. By allowing the mass spectrometer to scan exclusively at preselected ions (selected ion monitoring (SIM)), exceptionally high sensitivity and selectivity may be achieved.[6]

Quantitative mass spectrometry involving SIM has, however, not been evaluated systematically in moth pheromone work. One of the very few studies related to pheromones was performed by Buser and Arn.[7] These authors combined high resolution gas chromatography with SIM using electron ionization (EI). Since then the instrumentation available for mass spectrometry has improved in several important ways, such as allowing access to chemical ionization (CI) on a routine basis.

We have evaluated the usefulness of SIM of moth pheromone acetates using EI and CI. These acetates represent the most commonly occurring group of pheromone components (60–70%) among moths (see for example Ref. 8).

EXPERIMENTAL

Reagents

Solvents used were of analytical grade and not redistilled before use with the exception of *n*-heptane. Synthetic reference compounds used were from the laboratory's collection. Unless otherwise stated the chemical purity was ≥98%.

The acetates were dodecyl acetate (12:Ac), *Z*-7-dodecenyl acetate (*Z*7–12:Ac), *E,E*-8,10-dodecadienyl acetate (*E,E*-8,10–12:Ac), *Z*-9-tetradecenyl acetate (97%) (*Z*9–14:Ac), *Z,E*-9,11-tetradecadienyl acetate (*Z,E*-9,11–14:Ac) and *Z,E*-9,12-tetradecadienyl acetate (*Z,E*-9,12–14:Ac).

The alcohols were: Decanol (10:OH), *Z*-5-decenol (*Z*5–10:OH), *Z*-7-dodecenol (*Z*7–12:OH), *E,E*-8,10-dodecadienol (*E,E*-8,10–12:OH), *Z*-9-tetradecenol (*Z*9–14:OH), and *Z,E*-9,11-tetradecadienol (*Z,E*-9,11–14:OH).

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Acetic anhydride (99+at% ^2H) was purchased from Aldrich Europe, Beerse, Belgium.

Preparation of deuterated internal standards

To 50–100 mg of respective alcohol (10:OH, *Z*5–10:OH, *Z*7–12:OH and *Z*9–14:OH) dissolved in 0.2–0.5 ml of dry pyridine in a teflon-lined screw cap test tube was added 200–300 mg of acetic acid anhydride-($^2H_6$). After flushing with nitrogen the tube was closed and heated at 80 °C for 1 h. Ether and 1 M HCl were added and the organic phase subsequently washed with water, 5% $NaHCO_3$ and water. After drying (mol. sieve 5 Å) and filtration, the solution was evaporated to dryness. The residue, dissolved in a small volume of *n*-hexane, was chromatographed on 3 g of silicic acid (Mallinckrodt SilicAR CC4 299–325 mesh). The deuterated acetate was eluted with 10 ml of *n*-hexane followed by 8 ml of ether:hexane (1:10 v/v). The fractions of a purity >99% as indicated by gas chromatography were combined and evaporated to dryness. The yield was approximately 75% for each compound.

Gas chromatography mass spectrometry (GC/MS)

Analyses were performed on a Ribermag R10-10c quadrupole GC/MS/DA system equipped with a Carlo Erba model 4160 gas chromatograph. For quantitative analysis with deuterium-labelled analogues as internal standards, a 25 m fused silica column, i.d. 0.2 mm, deactivated with Carbowax 20M (280 °C) and dynamically coated with AT 1000 (Alltech Ass. Inc., Deerfield, Illinois, USA) as stationary phase was used. The rest of the separations were performed on a persilylated column coated with SE-54 (Alltech). The helium carrier gas velocity was 40 cm s^{-1}.

In all analyses a 1–3 µl sample was injected splitless at an injector temperature of 250 °C and a column temperature of 60 °C. The split valve was opened 50 s after injection and after 2 min the column temperature was increased linearly by 10 °C min^{-1} to 200 °C.

The electron energy used for EI was 25 eV or 70 eV and for CI 70 eV. The temperatures in the ion source were 140 °C (EI) and 110 °C (CI). Methane or ammonia at 1 Torr served as reactant gases.

Insect material

Turnip moths, *Agrotis segetum* (Schiff.) (Lepidoptera: Noctuidae) were raised in the laboratory[9]. Insects used for preparation of the pooled extract described below were the second generation offspring from insects collected in the field as pupae. Insects used for quantitative studies were from the fifth to sixth generation of the same culture.

Preparation of extracts

A pooled abdominal tip extract in hexane was prepared from 2–5-day old female turnip moths within a 2 h period of maximum calling activity.[9] Individual pheromone gland extracts were prepared in 8 µl of heptane with deuterated internal standard (200 picogram each of 10:Ac-($^2H_3$), *Z*5–10:Ac-($^2H_3$), *Z*7–12:Ac-($^2H_3$) and *Z*9–14:Ac-($^2H_3$) μl^{-1}) added.

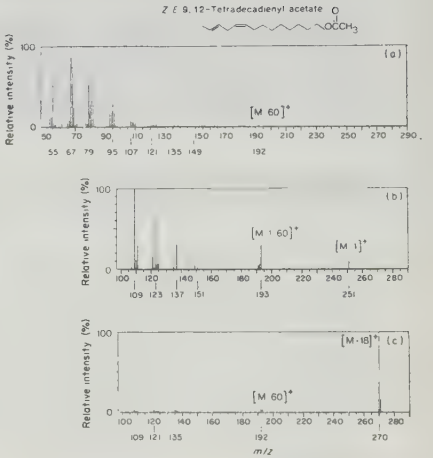

Figure 1. Mass spectra of *Z,E*-9,12-14:Ac. (a) EI 70 eV temperature in ion source 150 °C, (b) CI Methane and (c) CI Ammonia temperature in ion source 110 °C for both.

RESULTS AND DISCUSSION

Mass spectra of synthetic compounds

The EI and CI spectra of a series of saturated and unsaturated alcohols and acetates were studied. As an example mass spectra of *Z,E*-9,12-tetradecadienyl acetate are shown in Fig. 1.

In EI (Table 1), none of the investigated compounds gave abundant fragments in the high mass range. Molecular ions were produced only by the compounds possessing conjugated double bonds. In saturated, monounsaturated and diunsaturated acetates with isolated double bonds the peak of highest mass was found at m/z $[M-60]^+$ (loss of acetic acid) (cf. Refs 10–12). Cleavages related to those producing $[M-60]^+$ ions but with charge retention on the oxygen-containing units produced ions of m/z 61. For dodecyl acetate this peak was very intense (98%) but the intensity decreased significantly for the acetates with unsaturation. Hydrocarbon fragments of no diagnostic value form the base peaks in all these mass spectra.

Decreased energy of the bombarding electrons (25 eV) doubled the relative abundances of the $[M]^{+\cdot}$ or $[M-60]^+$ ions, but led to significant reduction in the total number of ions. The total ion current produced in CI is less than that in EI. The ions produced are, however, dependent on the chemical nature of the reactant gas and the analyte. Using methane as the reactant gas (Table 2) all compounds produced ions related to

Table 1. Electron impact ionization (70 eV)

Compound	Mol weight	Most abundant ion			Characteristic fragments Other significant ions					
		Assignment	*m/z*	%	Assignment	*m/z*	%	Assignment	*m/z*	%
12:Ac	228	C_5H_9	69	100	$[M-ACOH]^+$	168	10	$[ACOH+H]^+$	61	98
*Z*7–12:Ac	226	C_5H_7	67	100	$[M-ACOH]^+$	166	15	$[ACOH+H]^+$	61	5
E,E-8,10–12:Ac	224	C_5H_8	68	100	$[M]^{+\cdot}$	224	10	$[ACOH+H]^+$	61	5
E,E-8,10–12:OH	182	C_5H_8	68	100	$[M]^{+\cdot}$	182	10	—	—	—
*Z*9–14:Ac	254	C_5H_7	55	100	$[M-ACOH]^+$	194	10	$[ACOH+H]^+$	61	10
Z,E-9,12–14:Ac	252	C_5H_7	67	100	$(M-ACOH]^+$	192	5	$[ACOH+H]^+$	61	6
Z,E-9,11–14:Ac	252	C_5H_7	67	100	$[M]^{+\cdot}$	252	5	$[ACOH+H]^+$	61	3
Z,E-9,11–14:OH	210	C_5H_7	67	100	$[M]^+$	210	10	—	—	—

Table 2. Chemical ionization, CH_4

	Mol weight	Most abundant ion			Characteristic fragments Other significant ions					
		Assignment	*m/z*	%	Assignment	*m/z*	%	Assignment	*m/z*	%
12:Ac	228	C_8H_{15}	111	100	$[M+H]^+$	229	15	$[M+H-ACOH]^+$	169	50
Z7–12:Ac	226	C_8H_{15}	111	100	$[M+H]^+$	227	3	$[M-H-ACOH]^+$	165	20
E,E-8,10–12:Ac	224	C_9H_{13}	109	100	$[M]^{+\cdot}$	224	8	$[M+H-ACOH]^+$	165	15
E,E-8,10–12:OH	182	C_8H_{13}	109	100	$[M]^{+\cdot}$	182	15	$[M+H-H_2O]^+$	165	15
*Z*9–14:Ac	254	C_8H_{15}	111	100	$[M+H]^+$	255	5	$[M-H-ACOH]^+$	193	15
Z,E-9,12–14:Ac	252	C_8H_{13}	109	100	$[M-H]^+$	251	20	$[M+H-ACOH]^+$	193	35
Z,E-9,11–14:Ac	252	C_8H_{13}	109	100	$[M]^{+\cdot}$	252	20	$[M+H-ACOH]^+$	193	50
Z,E-9,11–14:OH	210	C_8H_{13}	109	100	$[M]^{+\cdot}$	210	20	$[M+H-H_2O]^+$	193	10

Table 3. Chemical ionization, NH_3

Compound	Mol weight	Most abundant ion			Characteristic fragments Other significant ions					
		Assignment	*m/z*	%	Assignment	*m/z*	%	Assignment	*m/z*	%
12:Ac	228	$[M+NH_4]^+$	246	100	$[M+H]^+$	229	8			
*Z*7–12:Ac	226	$[M+NH_4]^+$	244	100	$[M+H]^+$	227	3			
E,E-8,10–12:Ac	224	$[M+NH_4]^+$	242	100	—	—	—	$[M+H\ \ ACOH]^+$	165	10
E,E-8,10–12:OH	182	$[M+NH_4]^+$	200	100	$[M]^+$	182	3			
*Z*9–14:Ac	254	$[M+NH_4]^+$	272	100	—	—	—	$[M-ACOH]^+$	194	4
Z,E-9,12–14:Ac	252	$[M+NH_4]^+$	270	100	—	—	—	$[M-ACOH]^+$	192	5
Z,E-9,11–14:Ac	252	$[M+NH_4]^+$	270	100	$[M+H]^+$	253	5	$[M+H-ACOH]^+$	193	10
Z,E-9,11–14:OH	210	$[M+NH_4]^+$	228	100	$[M+H]^+$	211	5	$[M+H-H_2O]^+$	193	10

$[M]^{+\cdot}$ by charge transfer, hydride abstraction or protonation. However, extensive fragmentation also took place as indicated by the presence of ions formed by loss of acetic acid or water from the molecular adducts and by the presence of abundant hydrocarbon-type ions (base peak).

Very little fragmentation occurred when ammonia was used as reactant gas. Thus, the spectra of all compounds studied exhibited the adduct ion $[M+18]^+$ as the base peak (Table 3). Due to the weak acid character of the NH_4^+ ion very little protonation occurred.

SIM of synthetic compounds—evaluation of sensitivity

Ions for SIM as chosen from Tables 1–3 were investigated to determine the detection limit for the series of reference compounds (Table 4). The two mono-unsaturated acetates (*Z*7–12:Ac and *Z*9–14:Ac) and the diunsaturated *Z,E*-9,12–14:Ac (isolated double bonds) yielded the best signal to noise ratio (S/N) (at $m/z[M+18]^+$) with CI and ammonia as the reagent gas. Contrary to this the two alcohols (*E,E*-8,10–12:OH and *Z,E*-9,11–14:OH) showed far better S/N in EI than in CI. In the case of the saturated 12:Ac the best S/N was obtained when m/z 61 was monitored using electron impact ionization at low energy (25 eV). However, low energy EI did not improve the S/N for ions of high masses for example $[M]^{+\cdot}$, $[M-18]^+$ or $[M-60]^+$. This result is in contrast to that of Buser and Arn who recommended low electron energy to improve the sensitivity of mass fragmentographic analyses of moth pheromone acetates.[7]

We conclude that in the case of the acetates there are no significant differences between CI using ammonia as the reactant gas and EI with respect to sensitivity. It is

Table 4. Signal to noise ratios obtained for various ions in SIM of 100 pg of pheromone components: stationary phase; AT-1000

	EI 70 eV			EI 25 eV			CI-Methane	CI Ammonia
	61.10[a]	82.10[a]	$[M]^{+\cdot}$, $[M-18]^{+}$ b $[M-60]^{+}$	61.10[a]	82.10[a]	$[M]^{+\cdot}$, $[M-18]^{+}$ b $[M-60]^{+}$	$[M+1]^{+}$, $[M+1-60]^{+}$ c $[M(+1)-18]^{+}$	$[M+18]^{+}$
12:Ac	35	17	12	48	11	9	5	37
*Z*7–12:Ac	9	22	26	5	32	15	3	47
E,E-8,10–12:Ac	3	9	27	2	4	4	2	14
E,E-8,10–12:OH	1	2	2	1	1	1	1	1
*Z*9–14:Ac	3	13	9	2	10	3	1	15
Z,E-9,12–14:Ac	10	12	6	5	4	2	1	13
Z,E-9,11–14:Ac	2	18	12	1	6	2	1	11
Z,E-9,11–14:OH	1	6	8	1	4	1	1	1

[a] m/z 61.10 and m/z 82.10 monitored in multiple ion mode (2 ions).
[b] $m/z[M]^{+\cdot}$, $[M-18]^{+}$ or $[M-60]^{+}$ monitored in multiple ion mode (2 ions) as chosen from Tables 1 and 2. Only ion with highest S/N given.
[c] $m/z[M+1]^{+}$, $[(M+1)-60]^{+}$ or $[(M+1)-18]^{+}$ monitored in multiple ion mode (2 ions) as chosen from Table 3. Only ion with highest S/N given.

also indicated that diunsaturated alcohols should preferably be determined by monitoring the molecular ion in EI. Hogge and Olson demonstrated extremely sensitive recordings of full mass spectra of aliphatic alcohols using negative ion CI mass spectrometry with the corresponding heptafluorobutyrates.[13] This is an approach that may be used to advantage also in SIM.

SIM of a pooled moth pheromone extract

The *Agrotis segetum* female pheromone gland extract has been characterized by Löfstedt *et al.*[9] Thus it was known that the monitored quantities of *Z*5–10:Ac (**1**), 10:Ac (**2**), *Z*7–12:Ac (**3**), 12:Ac (**4**) and *Z*9–14:Ac (**5**) amounted to approximately 60, 80, 1000, 50 and 490 pg, respectively (Fig. 2). The quantitative relationships between the peaks differ in the various fragmentographic modes (Fig. 2(a)–(d). Thus monitoring at m/z 61.10 plus 82.10 in EI gave comparatively strong signals for the saturated acetates (compounds **2** and **4**) due to significant contribution from ions of m/z 61.10 (cf. Tables 1 and 4), while ions of m/z 82.10 make up most of the signal indicating the unsaturated compounds.

Fragmentation in EI leading to ions of m/z 82.10 occurs frequently with unsaturated compounds, no only acetates. Thus SIM assays based on monitoring this ion are rather unspecific and very sensitive to contamination.

The situation should be more favourable when fragments of higher masses $[M-18]^{+}$ and $[M-60]^{+}$ are monitored. Unfortunately, these ions are of low abundance in EI which results in poor sensitivity. Fragmentation by loss of acetic acid from the acetate and/or water from the corresponding alcohol, respectively, leads to identical ions. SIM of such ions is therefore valuable for the simultaneous analysis of acetates and alcohols. Thus, one of these compounds eluting prior to compound **3** could be *Z*7–12:OH, earlier identified in turnip moth extracts.[9] However, it is evident that additional compounds, present in the 12-carbon chain region, are indicated by this fragmentation.

CI with methane as reactant gas leads, as expected, to poor signal-to-noise ratios and 12:Ac was not even detected. On the other hand CI with ammonia as reactant gas produced very good signals and the procedure seems to be quite specific for the acetates.

We conclude therefore that CI with methane as reactant gas is of no use for SIM of acetates. EI and CI with ammonia as reactant gas are competitive in terms of sensitivity, with EI monitoring at m/z 61.10 being particularly advantageous for saturated acetates. However, the higher selectivity achieved for CI with ammonia as reactant gas should be borne in mind when complex samples are to be analysed.

Quantification

The isotopic purity of the ($^2H_3$)acetates was investigated by ammonia CI SIM. The two ions corresponding to $[M+18]^{+}$ of the ($^2H_3$)acetate and the unlabelled analogue (three mass units lower), were monitored simultaneously. Only a very small signal (<1%) was detected on the lower mass. Peak maximum coincided in time for the two ions. Since it was found that the gas chromatographic retention time on the capillary column used (AT 1000) for the deuterated acetate was significantly shorter than that of the unlabelled analogue, it may be assumed that the signal recorded at a nominal mass three units less is not due to isotopic impurities, but to $[M+15]$ cleavage fragments yielded by the labelled compound. Corresponding fragments were also observed in the case of the unlabelled acetate. It is at present not known whether these represent small reproducible contributions to the normal mass spectrum or if their occurrence is influenced by the conditions in the ion source. A similar phenomenon regarding long-chain alcohols was reported by Odham and Stenhagen.[21] Although of small magnitude, the signals at m/z $[M+15]^{+}$ should be carefully checked, as they define the blank level.

The plots relating the amount of analyte to the ion ratio m/z $[M+18]^{+}$ for analyte to m/z $[M+18]^{+}$ for labelled internal standard) showed correct linearity ($r=0.9970$ to 0.9989) (Fig. 4, Table 5). The observed *y*-intercept is explained by the low but detectable abundance of ions of $m/z[M+15]^{+}$ in the mass spectrum of the labelled standard. Ideally the slope should have been the same for all the compounds. The variation could be explained by slightly different concentrations of the stock solutions from which all the dilutions were made up in consecutive steps. The limit of detection (LOD)

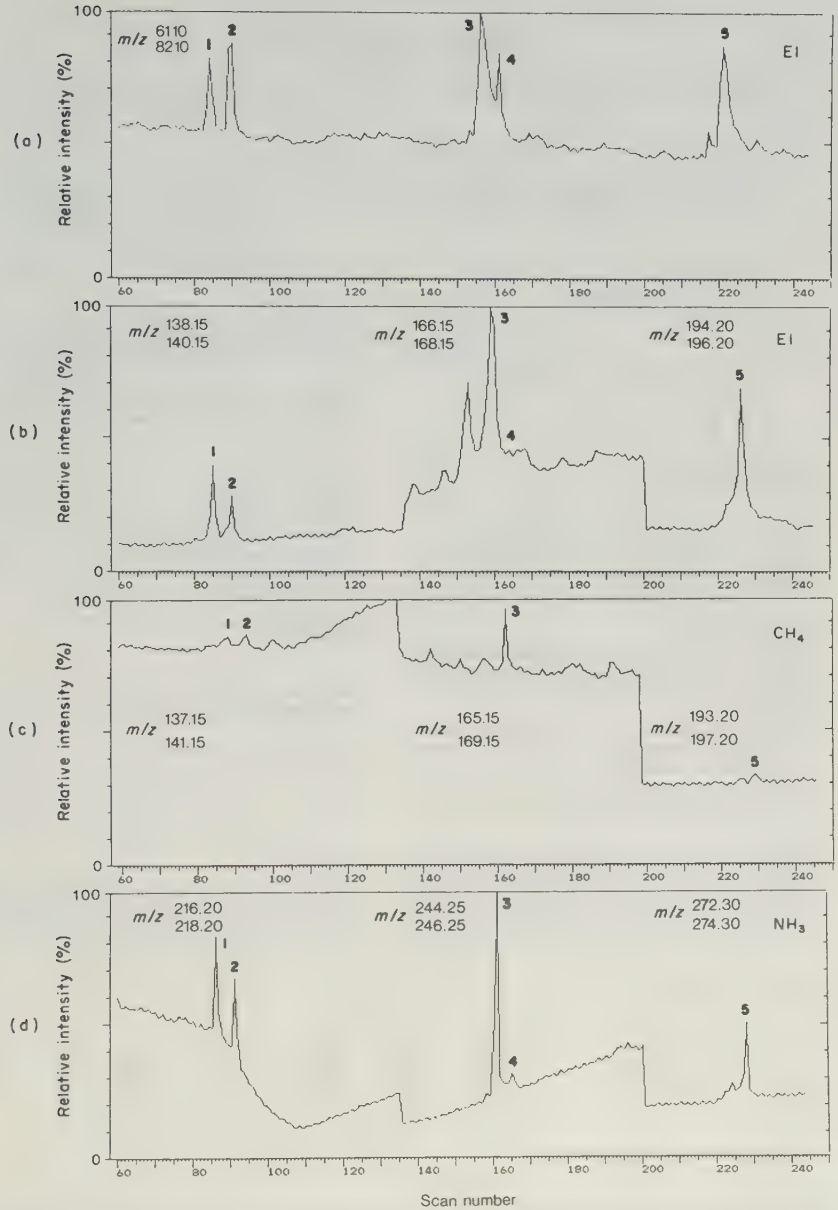

Figure 2. Mass fragmentogram (sum of selected ion signals) of a pooled *Agrotis segetum* female pheromone gland extract. (a) EI monitoring at the acetate characteristic m/z 61.10 fragment and at m/z 82.10. (b) EI monitoring masses in the high mass range ([M − 60] for acetates), (c) CI-Methane, (d) CI-Ammonia. Changes in the base line indicate changes in parameter sets during analyses. The numbers in the fragmentogram denote Z5–10 Ac (1), 10: Ac (2), Z7–12 Ac (3), 12: Ac (4) and Z9–14 Ac (5) corresponding to approximately 60, 80, 1000, 50 and 490 picograms, respectively. One female equivalent injected in each analysis

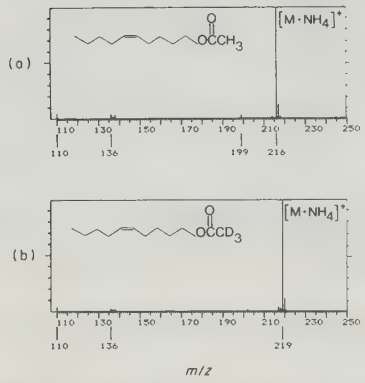

Figure 3. CI–NH_3 mass spectra of *Z*5–10: *Ac*(a) and *Z*5–10: Ac-($^2H_3$) (b). 70 eV, 110 °C in ion source.

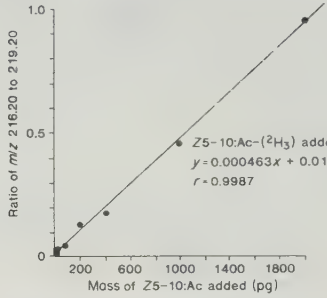

Figure 4. Plot relating amount of *Z*5–10: Ac added to 2 µl heptane samples containing 5.0 pg of internal standard (*Z*5–10: Ac-($^2H_3$)) to the *m*/*z* 216.20 to *m*/*z* 219.20 ion ratio.

and limit of quantification (LOQ), as defined by Ref. 14, were calculated to be 43–72 pg and 144–247 pg, respectively. This conservative LOD can be compared with, for example, the undefined limit of detection ('better than ... 100 pg') reported for *E,Z*-7,9-dodecadienyl acetate.[7]

The coefficient of variation for determination of for example 2 ng *Z*7–12: Ac was calculated[15] to be 4% while this figure increased to about 12% when the amount was 500 pg.

The frequently observed discrimination in splitless capillary gas chromatography is a problem in quantitative work (see for example Ref. 17). The discrimination should, however, be very small in the case of deuterated internal standards having chemical and physical properties almost indentical with those of the unlabelled analogues.

Although deuterated internal standards might be ideal in many aspects their use requires monitoring of at least two ions in SIM. It is generally recognized that the precision of quantitative SIM decreases with the number of ions monitored.[18] For this reason compounds chromatographically well-resolved from the analyte allowing monitoring on one ion in different parameter sets should be tried out as alternative internal standards. In principle, such an approach implies 'single ion monitoring' being the ultimate with respect to both sensitivity and precision.

APPLICATION

Effect of daytime and mating on pheromone production in the turnip moth *Agrotis segetum*

Pheromone titre is known to vary with environmental and physiological parameters in moth species (for a review, see Ref. 19). However, the precise relationship between, for example, calling activity and pheromone titre has not been established. Neither has a general relation between mating history and pheromone titre been documented.

The pheromone of *A. segetum* is a three component mixture of *Z*5–10: Ac, *Z*7–12: Ac and *Z*9–14: Ac[9,20] while 10: Ac might play a subtle role in close-range behaviour[9] although no increase in trap catches due to

Table 5. Regression data

Analyte	Internal standard	*n*	slope (β)	Intercept (α_0)	Σx_i^2	$\Sigma(Y-\hat{Y})^2$	*r*	LOD(pg)	LOQ(pg)
10: Ac	10: Ac-($^2H_3$)	8	0.9705	0.0098	0.8612	$15.5 \cdot 10^{-4}$	0.9989	43	144
*Z*5–10: Ac	*Z*5–10: Ac-($^2H_3$)	8	0.9270	0.0123	0.8612	$35.7 \cdot 10^{-4}$	0.9987	69	229
*Z*7–12: Ac	*Z*7–12: Ac-($^2H_3$)	8	1.0243	0.0115	0.8612	$47.9 \cdot 10^{-4}$	0.9970	72	240
*Z*9–14: Ac	*Z*9–14: Ac-($^2H_3$)	8	0.8459	0.0217	0.8612	$33.0 \cdot 10^{-4}$	0.9973	72	247

$\Sigma x_i^2 = \Sigma (X - \bar{X})^2$

$\hat{Y}$ = *Y*-value estimated from the regression

$LOD = \alpha_0 + 3s_{\alpha_0}$ [14]

$LOQ = \alpha_0 + 10s_{\alpha_0}$ [14]

$$s_{\alpha_0} = s_{xy}\sqrt{\frac{1}{n} + \frac{\bar{x}^2}{\Sigma x_i^2}}$$ [16]

$$s_{xy} = \sqrt{\frac{1}{n-2}\Sigma(Y-\hat{Y})^2}$$

Table 6. Quantitative analyses of individual *A. segetum* females

	Insects	Time of sampling[a]	Picogram of respective compound ±S.D. (no. of individuals) 10:Ac	Z5–10 Ac	Z7–12 Ac	Z9–14 Ac
I	Virgin female (extract)	4–5 h into scotophase	206 ± 117 (6)	439 ± 222 (6)	2347 ± 2034 (6)	1091 ± 1683 (4)
II	Virgin female (extract)	6–7 h into photophase	112 ± 51 (4)	284 ± 217 (4)	1218 ± 810 (4)	674 ± 667 (3)
III	Mated female (extract)	4–5h into scotophase, the day after mating	523 ± 740 (6)	145 ± 91 (6)	491 ± 440 (6)	335 ± 360 (5)
	Statistics, pair-wise comparisons	I–II one-sided[b] *t*-test	$p = 0.1$	NS	NS	NS
		II–III two-sided *t*-test	NS	NS	NS	NS
		I–III one-sided[c] *t*-test	NS	$0.02 > p > 0.01$	$0.10 > p > 0.05$	NS

[a] Diurnal rhythm of calling was described by Löfstedt *et al.* (1982).
[b] The hypothesis to be tested was that female pheromone content should be lower during non-calling hours.
[c] The hypothesis to be tested was that female pheromone content should decrease after mating.

addition of this compound could be seen (Löfstedt & Löfqvist, unpublished). The content of pheromone components was determined in individual 4–5-day old *A. segetum* females (Table 6). Abdominal tip extracts were prepared from virgin females at time of maximum calling (4–5 h into the scotophase) and 6–7 h into the photophase. Mated females were sampled 4–5 h into scotophase the day after mating. We expected the pheromone titre to be lower during the photophase, relative to hours of sexual activity. However, the decreased amounts of *Z*5–10: Ac and *Z*7–12: Ac after mating were the only changes that were statistically significant. Within the various treatments the standard deviation of the quantities observed (Table 6) can be compared with the precision of the assay as described above. For example, the amount of *Z*5–10: Ac per virgin female during photophase is 439 ± 222 pg compared to a SD of ±60 pg at the 500 pg level. In this case one fourth of the 'individual variation' is method error. But in the case of *Z*7–12: Ac the SD at the 2000 pg level

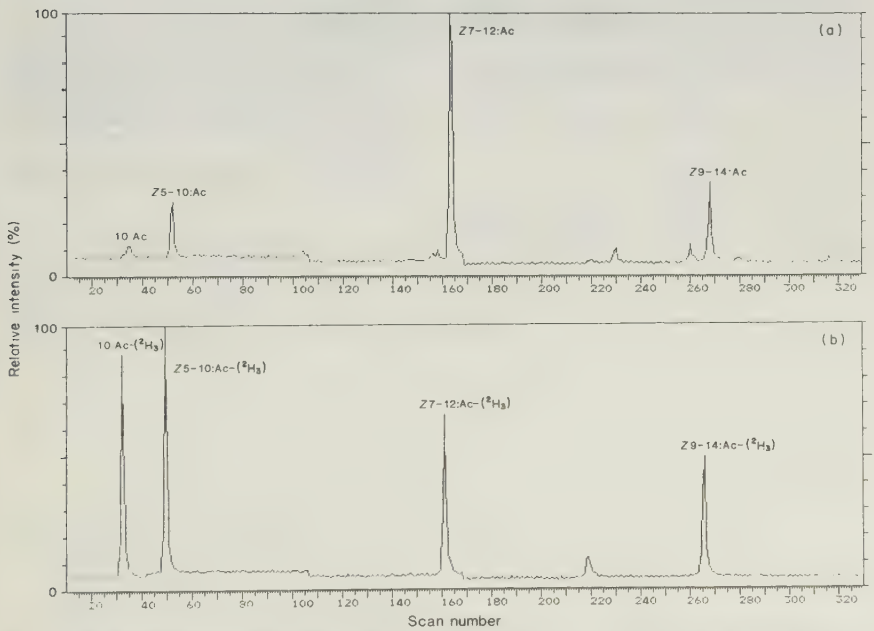

Figure 5. Quantitative analysis of an *Agrotis segetum* female pheromone gland extract (a) Mass fragmentogram monitoring at *m/z* 218.20, 216.20, 244.25 and 272.30 showing compounds to be quantified (b) Mass fragmentogram monitoring at *m/z* 221.20, 219.20, 247.25 and 275.30 showing deuterated internal standards. The signals in (b) correspond to 1.6 ng of each component

(80 pg) is less than 4% of the observed SD of the sampled population.

The small rather than dramatic decrease in pheromone titre due to mating, corresponds with a general hypothesis about pheromone titre and mating history.[19] In species like *A. segetum* that frequently mate more than once, no pronounced decrease in pheromone content should be observed after mating. In contrast, females that never mate twice may exhibit rapid decline in pheromone titre as soon as they are mated.

CONCLUSIONS

Quantitative mass spectrometry using SIM with CI and ammonia as reactant gas enables studies of moth pheromone acetates in the picogram range. The analytical procedure as presented here can probably be refined with respect to precision but it already provides a fast and convenient approach to quantitative analysis at subnanogram levels. As demonstrated by the application, such analyses open possibilities of studies of pheromone composition in individuals of many more moth species.

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INDIVIDUAL VARIATION IN THE PHEROMONE OF THE TURNIP MOTH *Agrotis segetum*[1), 2)]

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ABSTRACT

Female turnip moths (Agrotis segetum) from a laboratory culture inbred for more than 30 generations, and the offspring (1st to 3rd generation) from field collected insects were analysed individually for acetates and alcohols in the pheromone gland. Quantitative analysis of individual components was performed at the subnanogram level by gas chromatography-mass spectrometry (selected ion monitoring). The titre of the pheromone, i.e. the sum of the homologous acetates (Z)-5-decenyl acetate, (Z)-7-dodecenyl acetate and (Z)-9-tetradecenyl acetate was 2.0 ± 0.3 ng in the laboratory culture and 3.2 ± 0.6 ng in the "wild strain". There was no correlation between pheromone titre and female weight. The relative proportion of the pheromone components varied substantially between individuals, but there was no statistically significant difference between the two populations. The percentages of the respective compounds (x ± coefficient of variation) were for Z5-10:OAc 14.8 ± 127 %, for Z7-12:OAc 55.6 ± 32 % and for Z9-14:OAc 29.6 ± 59 %. The pheromone composition varied more in the wild strain than in the laboratory culture. The significance of the pheromone variation to the attraction of males was tested in a field experiment. The ratio of males trapped by the most attractive blend versus the least attractive one was 2.2.

INTRODUCTION

In general female moth sex pheromones have been found to be a mixture of two or more compounds required, in some cases as a precise blend to elicit full sexual response. A pheromone component missing in a synthetic blend or small changes in the ratio between compounds can affect the male behavioural response dramatically (Linn & Roelofs 1983). In tortricid moths the precisely regulated (Z)/ (E)-11 tetradecenyl acetate ratio also seems to be one of the major mechanisms by which reproductive isolation is maintained among sympatric species (Roelofs & Brown 1982).

There is not yet enough information available on withinspecies variation in pheromone production to generalize about its relation to mating success. Miller and Roelofs (1981) analysed almost 400 individual females of the red-banded leafroller, Argyrotaenia velutinana, known to use a mixture of (Z) and (E)-11-tetradecenyl acetate (Z11 and E11-14:OAc) in its pheromone. The Z/E-ratio was found to be precisely regulated to 91:9, which is close to the ratio found to be maximally attractive to males in the field (Roelofs et al. 1975). The European corn borer (Ostrinia nubilalis), a pyralid, also has Z11- and E11-14:OAc as main components of the pheromone. In nature at least two forms, with opposite ratios between the two isomers, coexist in some areas and, although hybrids are formed, the polymorphism is maintained. One probable mechanism for this is male preference for the pheromone of its own strain (Klun & Maini 1979, Cardé et al. 1978).

In the turnip moth, Agrotis segetum, (Noctuidae) the pheromone consists of at least three homologous monounsaturated acetates; (Z)-5-decenyl acetate

(Z5-10:OAc), (Z)-7-dodecenyl acetate (Z7-12:OAc) and (Z)-9-tetradecenyl acetate (Z9-14:OAc) (Löfstedt et al 1982, Arn et al 1983). In general the three acetates are recovered from female abdominal tips in quantities between 0.05 and 2 ng per female. In addition to these compounds the gland extracts contain at least another 10 compounds chemically related to the behaviourally active pheromone components (Löfstedt et al 1982). The field screening tests of Arn et al. (1983) showed that baits with a wide range of ratios between the three homologous acetates were equally attractive to male turnip moths. This suggested to us that the pheromone production in the turnip moth females should not be precisely regulated. In the present study individual variation in pheromone production in two strains of turnip moths was quantified by gas chromatography-mass spectrometry in the selected ion monitoring mode (SIM). One strain was inbred for more than 30 generations and the other was 1st to 3rd generation offspring from insects collected as pupae in the field. Field trapping experiments were carried out to obtain a rough estimate of the biological significance of the variation to "mating success", as measured by trapping activity.

METHODS AND MATERIALS

Insect material. The laboratory strain of Agrotis segetum, at the time of this study inbred for more than 30 generations, was earlier described by Löfstedt et al (1982). A "wild strain" was initiated from about 20 wild insects collected as pupae in southernmost Sweden in the autumn of 1981. Insects analysed from this strain were 1st to 3rd generation offspring of the field collected specimens. All insects were maintained as described by Löfstedt et al. (1982) on a reversed 16:8 h light:dark cycle.

Collection of pheromone gland extracts. From time of emergence until analysis the insects were kept individually in 250 ml plastic jars and fed a 5 % sucrose solution. Ovipositor extracts were prepared from 2-6 day old females. The pheromone titre shows only a minor age dependence within this range (Löfstedt, unpublished). The extraction was performed at the time of maximum calling activity (pheromone gland eversion) ca 4 hr into the scotophase (Löfstedt et al. 1982) by soaking the tip in 7 µl heptane with internal standard added. The internal standard was a series of straight-chain acetates including nonyl acetate (9:OAc), undecyl acetate (11:OAc), tridecyl acetate (13:OAc) and pentadecyl acetate (15:OAc), added in 250 pg each per µl solvent. The samples were placed in the freezer for about one day after which the solvent was decanted and transferred to a glass ampoule that was sealed and stored in the dark at -20°C until analysis. Insects were weighed (dry weight, 105°C, 24 h) after the abdominal tip had been removed.

Selected Ion Monitoring (SIM). The insect extract, between 2 and 4 µl recovered from each female, was injected splitless into a Finnigan 4021 gas chromatograph-mass spectrometer equipped with an Incos computer system. The capillary columns used were coated with Superox FA (Supelco Inc.), a polyethylene glycol type stationary phase. Insects from the laboratory culture were analysed on a WCOT glass column (62 m x 0.25 - 0.30 mm id) with a film thickness of 0.31 µm, while a WCOT fused silica column (25 m x 0.2 mm id) with a film thickness of 0.37 µm was used for the wild insect offspring. The linear helium flow through the columns was 22 cm/s at 60°C. The injector temperature was 210°C, and the split valve was opened 0.5 min after injection. The column temperature was maintained at 60°C for 5 min following injection, and then heated to 230 at 10°C/min.

The electron energy used was 60 eV (optimized) and the temperature in the ion source 250°C. The separations were monitored by focusing the mass spectrometer on fragments m/z 61 and m/z 82. Mass windows used were m/z 60.6 - 61.4 and 81.6 - 82.4 and the scanning times were 0.178 and 0.349 s. for the laboratory and the "wild strain" insects, respectively. The width of the peaks at half height was 3-4 s., which gave at least 8 scans per peak. In the course of the insect extract analyses, the identities of the specific compounds were checked by comparison with retention times of synthetic references.

For quantification, the intensities of m/z 61 and m/z 82 were summarized in each scan and the areas of the GC-peaks were determined. These areas were compared with the mean value of the areas of the internal standards. The response factors used in these comparisons were based on analyses of a dilution series of synthetic reference compounds with a fixed amount of the internal standard added. The calibration curve for each compound so obtained was based on 16-20 points (see Table 1). The relative abundance of m/z 61 and m/z 82 varies among the compounds studied (Löfstedt & Odham 1984, Lanne et al. 1984), therefore, the response factors of the compounds of interest, relative to the saturated acetates used as internal standards, ranged between 0.325 and 0.947.

Limit of Detection (LOD = background signal + three times the standard deviation) and Limit of Quantification (LOQ = background signal + ten times the standard deviation) (Anonymous 1982) were conservative and calculated as described in Löfstedt & Odham (1984). A low background signal and a high correlation coefficient of the calibration curve result in low values for LOD and LOQ. For the quantification of constituents of the internal standard (9:OAc, 11:OAc, 13:OAc and 15:OAc) throughout the whole study, the coefficient of variation did not exceed 7 % for any of the compounds.

To obtain an overview of the volatile contents of the individual insects, multivariate data analysis was applied according to Wold et al. (1984). The chemical data from each female was treated as a point in a p-dimensional

Table 1. Data from calibration curves for SIM-analysis of moth pheromone gland constituents

Compound	Response factor[1)]	Coeff. of correlation	LOD ng[2)]	LOQ ng[2)]
10:OAc	.82 ± .05	.997	.03	.09
12:OAc	.94 ± .03	.995	.22	.32
14:OAc	.92 ± .05	.994	.21	.30
Z5-10:OAc	.63 ± .06	.988	.09	.21
Z7-12:OAc	.91 ± .10	.982	.22	.45
Z9-14:OAc	.95 ± .11	.981	.21	.43
Z5-10:OH	.32 ± .03	.984	.05	.11
Z7-12:OH	.44 ± .05	.979	.11	.21
Z9-14:OH	.48 ± .07	.963	.14	.28

1) 95 % confidence interval

2) For calculation of LOD (Limit of Detection) and LOQ (Limit of quantification): see Löfstedt & Odham (1984).

space, where p is the number of quantified compounds 10:OAc, Z5-10:OAc, 12:OAc, Z7-12:OAc, Z7-12:OH, Z9-14:OAc and Z9-14:OH. A log (x + 0.001) transformation of the titres of the seven compounds was performed. With principal component analysis of such a data set, a 2-dimensional window into the 7-dimensional space is calculated in such a way that it shows as much as possible of the spread of the data. Field tests. Field trapping experiments were carried out in carrot and sugar beet fields in the province of Skåne, southern Sweden. In a first experiment thirteen different blends of 10:OAc, Z5-10:OAc, Z7-12:OAc and Z9-14:OAc were prepared as to cover most of the observed range of ratios between the unsaturated compounds. Because of the unclear behavioural function of 10:OAc the amount of this compound was held constant at 1.25 µg per bait. The other compounds were varied as proportions of a total 24 µg. Stock solutions of the blends were made up in a mixture of pentane and hexane. The blend in 25 µl solvent was applied to a 1 ml polyethylene capsule with 1.5 mm wall thickness (Kartell, Italy). The solvent was evaporated at room temperature and the capsule was closed. The baits were usually put in the traps the same day they were made, but were occasionally stored 1-2 days in the freezer (-20°C) prior to use.

The sticky wing traps used (Albany Inc., Needham Heights, Mass., U.S.A.) were checked daily and the baits were renewed. This step was considered to be important as the compounds of the pheromone have quite different volatilities.

Four replicate series of traps (blocks) were run for 6 nights. Within each series the traps were spaced at least 20 m apart and placed in a row at a right angle to what was presumed to be the predominant wind direction. The distance between series was at least 300 m.

In a second series of experiments the absolute amount of one of the homologous acetates was varied over two orders of magnitude, whereas the amounts of the remaining two were kept constant. The standard bait used in this experiment was a 1:5:2.5 mixture of Z5-10:OAc, Z7-12:OAc and Z9-10:OAc. The experiment was performed at two dosages with 0.3 and 3 µg respectively of the Z5-10:OAc in the standard bait. Stock solutions of the blends were made up in hexane. The blend in 100 µl solvent was applied to a rubber septum (A.H.Thomas Company, Philadelphia, Pennsylvania). The solvent was evaporated at room temperature and the dispenser was then stored at -20^{o} C until it was used. The sticky traps used were made in our laboratory. They had a 4 cm opening in all directions, a 20 x 20 cm^{2} sticky bottom and a cover of the same size (Löfqvist & Jönsson, unpublished). Five replicate series of traps (blocks) were spaced out and placed as in the first experiment described above. Chemicals. Compounds for the field experiments were at least 99 % pure regarding geometrical and positional isomers and the overall chemical purity was more than 98 %. 10:OAc was purchased from Schuchardt AG, München, FRG, and Z7-12:OAc and Z9-14:OAc from the Institute for Pesticide Research, Wageningen, The Netherlands. Z5-10:OAc was a gift from Bernt Thelin, Lund University, Sweden.

RESULTS

Chemical identification and quantification of pheromone components and related compounds in the gland extracts. The SIM-analyses were usually selective enough to provide neat tracings of individual tips and the blank showed an essentially smooth baseline (Fig. 1a and b). For positive identification of the chromatographic peaks, both the retention-index and the ratio of m/z 61 to m/z 82 had to correspond with those of the synthetic reference compounds. Only occasionally were there reasons to question the identity of a peak (see below for Z9-12:OAc).

The quantification report for each insect was compared to the calculated LOQ-values. Most values for Z7-12:OAc and Z9-14:OAc were above the LOQ. For the third behaviourally active monounsaturated acetate, Z5-10:OAc, about half of the values were above LOQ. For the remaining compounds (see Table 1) the quantitative reports should be considered approximate as only few values exceeded LOQ. Bar diagrams of the titre of 10:OAc, Z5-10:OAc, Z7-12:OAc and Z9-14:OAc in the individual females are displayed in Fig. 2. Most probably the

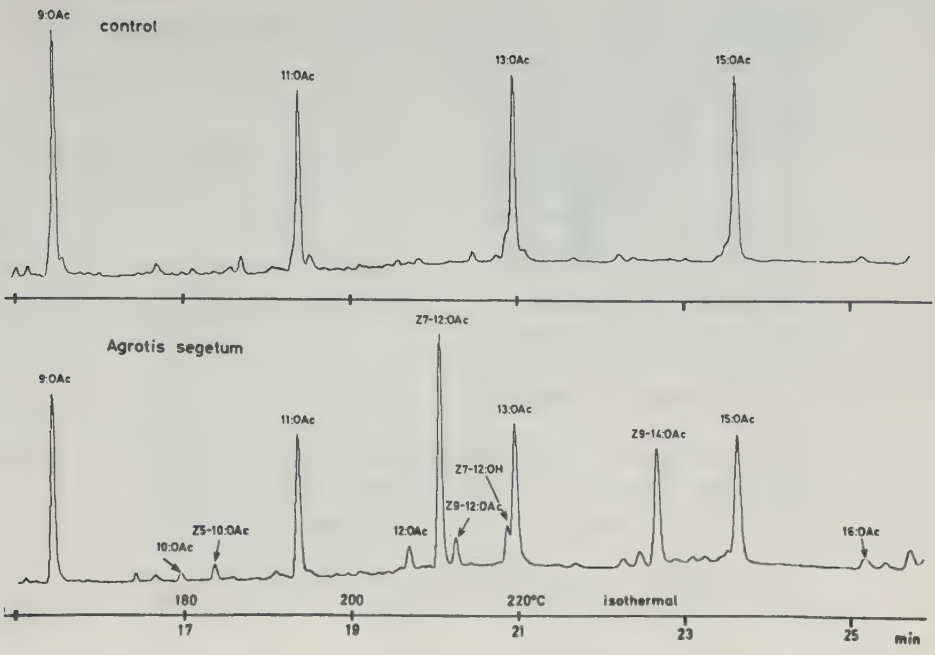

Figure 1. Reconstructed ion chromatograms (m/z 61+82) obtained from GC-MS analyses of a control (A) and an individual Agrotis segetum female ovipositor extract (B). The peaks of the internal standards correspond to 1.75 ng each.

low amount of the pheromone components did not contribute to the variation, as there was no correlation between the means of the quantified compounds in the two populations and their respective coefficients of variation ($r = 0.01$, $n = 15$).

Comparison of the laboratory culture and the "wild strain" insects. An overview comparison of the two populations revealed a higher spread in the composition of the pheromone gland secretions in the wild strain insects (Fig. 3). One extreme female containing no Z7-12:OAc and no Z9-14:OAc, was excluded from the data-set. The amounts of Z7-12:OAc and Z9-14:OAc were not significantly different in the two populations (Table 2). However, the amount of Z5-10:OAc was higher in the wild insects then in the insects from the laboratory culture, 0.35 ng and 0.17 ng respectively. Also the 10:OAc titre was higher in the "wild strain" insects than in the laboratory strain, but the difference was not significant. Because of the very skewed distribution of the 10:OAc titre in the "wild strain" population, a non-parametric test (Mann-Whitney U-test) was employed for the comparison. The compound earlier assigned as Z9-12:OAc (Löfstedt et al. 1982) was excluded from the comparison of the

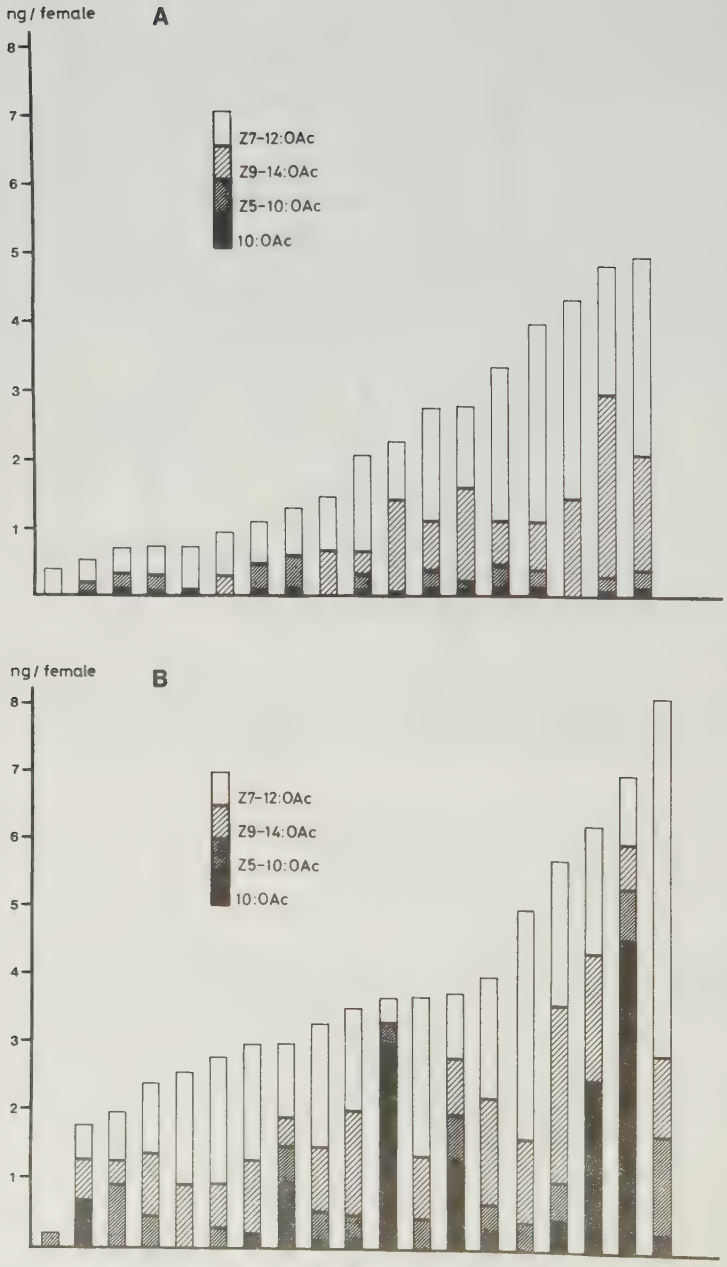

Figure 2. Absolute amounts of 10:OAc, Z5-10:OAc, Z7-12:OAc and Z9-14:OAc in ovipositor extracts from individual laboratory reared strain (A) and wild strain (B) Agrotis segetum females. The individuals are arranged in order of increasing pheromone titre.

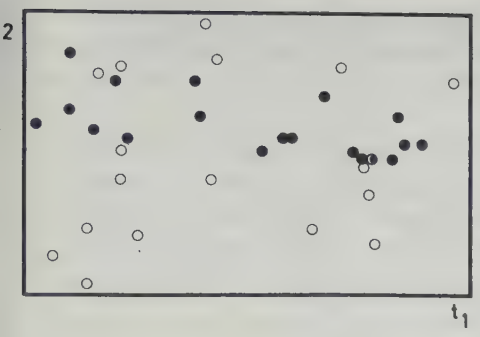

Figure 3. Principal component analysis of the titre of seven pheromone gland constituents (10:OAc, Z5-10:OAc, 12:OAc, Z7-12:OAc. Z7-12:OH, Z9-14:OAc and Z9-14:OH) in 37 Agrotis segetum females. The 37 points in the 7-dimensional space are projected onto a plane, chosen in such a way that the summed least square distances of the 37 points to the plane is minimized. ● = laboratory culture, O = wild strain insects. Most of the variation along the x-axis is due to variation in 10:OAc titre.

Table 2. Average amount of pheromone components and analogues in extracts of individual A.segetum females from a laboratory culture (n=18) and a wild strain of insects (n=19) [a]

Compound	Laboratory culture[b] $\bar{x}$ SE(ng)	Wild strain[c] $\bar{x}$ SE(ng)	Statistics (two-sided)	
10:OAc	0.07 ± 0.01	0.74 ± 0.29	P=0.43	Mann-Whitney U-test
Z5-10:OAc	0.17 ± 0.03	0.35 ± 0.06	P=0.02	Student's t-test
Z5-10:OH	d)	0.20 ± 0.04		--------
12:OAc	0.16 ± 0.02	0.09 ± 0.02	P=0.43	Student's t-test
Z7-12:OAc	1.20 ± 0.20	1.57 ± 0.26	P=0.26	- " -
Z7-12:OH	0.36 ± 0.06	0.40 ± 0.04	P=0.49	- " -
Z9-14:OAc	0.64 ± 0.16	1.32 ± 0.43	P=0.15	- " -
Z9-14:OH	0.18 ± 0.02	0.08 ± 0.03	P=0.02	- " -
Sum of behaviourally active acetates (Z5-10:OAc + Z-12:OAc + Z9-14:OAc)	2.01 ± 0.32	3.23 ± 0.57	P=0.07	- " -

a) All values were included, also those below LOQ

b) Inbred for more than 30 generations

c) 1st to 3rd generation offspring from insects collected in the field

d) Not quantified because of interference with other peak

two strains because of the drifting retention-times observed in the "wild strain" insects. It may be noticed that the relative composition of the gland extracts was very variable. In some females the amount of single compounds was below the limit of detection.

The sum of the behaviourally active mono-unsaturated acetates (Z5-10:OAc + Z7-12:OAc + Z9-14:OAc) was calculated for each individual moth. The higher average titre in the "wild strain" insects was not statistically significant (Table 2). The proportions of Z5-10:OAc, Z7-12:OAc and Z9-14:OAc respectively, were also calculated (Fig. 4a), but no statistically significant differences between the two populations were found (Table 3a). Thus the figures from both populations were pooled to calculate the mean proportion of the pheromone components for all females (n=38) and the correlation between the titres of the respective compounds (Table 3b). Whereas there was a significant positive correlation between the titres of Z7-12:OAc and Z9-14:OAc, Z5-10:OAc was not significantly correlated to either of Z7-12:OAc or Z9-14:OAc.

The average weight of the "wild strain" insects ($x \pm SEM$) was significantly higher than that of the laboratory culture, 126 ± 9 and 86 ± 6 mg respectively ($P = 0.001$, Student's t-test). There was no correlation between the dry weight

Table 3A. Relative pheromone gland titre (%) of pheromone components

	Laboratory strain (n=18)	Wild strain (n=19)	Two-sided T-test	Pooled data (n=38)		
Compound	$\bar{x} \pm SEM$	$\bar{x} \pm SEM$	(P-value)	$\bar{x}$	CV	CV(arcsin-trans
Z5-10:OAc	13 ± 3	17 ± 5	0.48	15	127%	83%
Z7-12:OAc	61 ± 3	50 ± 5	0.07	56	32%	27%
Z9-14:OAc	26 ± 4	33 ± 4	0.31	29	59%	41%

Table 3B. Correlation between titres of pheromone components, using pooled data from laboratory and wild strain (n=38)

Compounds	Coefficient of correlation (Pearson)	P
Z5-10:OAc with Z7-12:OAc	0.14	0.39
Z5-10:OAc with Z9-14:OAc	0.14	0.42
Z7-12:OAc with Z9-14:OAc	0.37	0.02

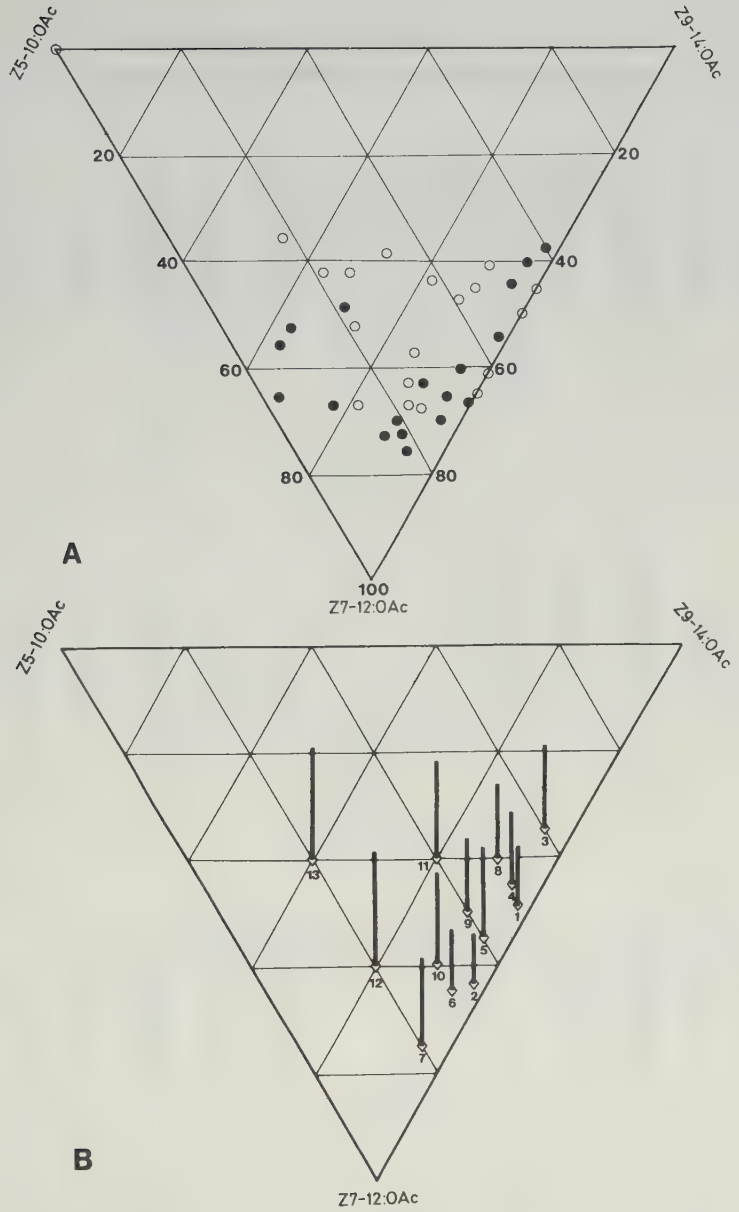

Figure 4. (A) Relative amounts of Z5-10:OAc, Z7-12:OAc and Z9-14:OAc in ovipositor extracts of individual Agrotis segetum females. ● = laboratory reared strain (n=18) and O = wild strain culture (n=19). In each corner of the triangle, the relative amount of one compound is 100 % and along the opposite side of the triangle the amount of this compound is 0 %. As an example the values for Z7-12:OAc are given in the figure. (B) Mean trap catches (n=6) of male A. segetum in a field experiment with different mixtures of Z5-10:OAc, Z7-12:OAc and Z9-14:OAc. For bait numbers and statistical data, see table 4.

Table 4. Mean trap catches (n=6) of male A.segetum in a field experiment with different proportions of pheromone components [1)]

	Amount in polyethylene cap (µg)				
Bait no	10:Ac	Z5-10:Ac	Z7-12:Ac	Z9-14:Ac	Mean catch[2)]
1	1.25	0.50	12.25	12.25	14.5 de
2	1.25	0.50	16.00	8.50	12.7 e
3	1.25	1.25	8.75	15.00	20.2 abcde
4	1.25	1.25	11.25	12.50	17.6 bcde
5	1.25	1.25	13.75	10.00	22.3 abcd
6	1.25	1.25	16.25	7.50	15.1 cde
7	1.25	1.25	18.75	5.0	21.7 abcd
8	1.25	2.50	10.00	12.50	18.2 bcde
9	1.25	2.50	12.50	10.00	17.9 bcde
10	1.25	2.50	15.00	7.50	22.7 abc
11	1.25	5.00	10.00	10.00	23.8 ab
12	1.25	5.00	15.00	5.00	28.2 a
13	1.25	10.00	10.00	5.00	27.8 a

1) cf Fig. 5

2) Means followed by the same letter are not significantly different by analysis of variance ($\sqrt{X+0.5}$) transformation) followed by Duncan's multiple range test ($P<0.05$)

of all the females (n=36) and their pheromone titre, i.e. the sum of Z5-10:OAc, Z7-12:OAc and Z9-14:OAc ($r = 0.26$, $P = 0.12$, Pearson).

Male response to pheromone blends of individual females in the field. The synthetic lures, approximately covering the observed variation in individual female pheromone composition, differed significantly in attractivity (Fig. 4b and Table 4). More than 1200 males were trapped during the experiment. The synthetic lure that comes closest to the average female pheromone composition (15 : 66 : 30), does not catch significantly different from the most attractive lure, (no. 10 vs. 12) (Table 4). There is a positive correlation between the amount of Z5-10:OAc in a bait and its attractivity ($r = 0.76$). In a second

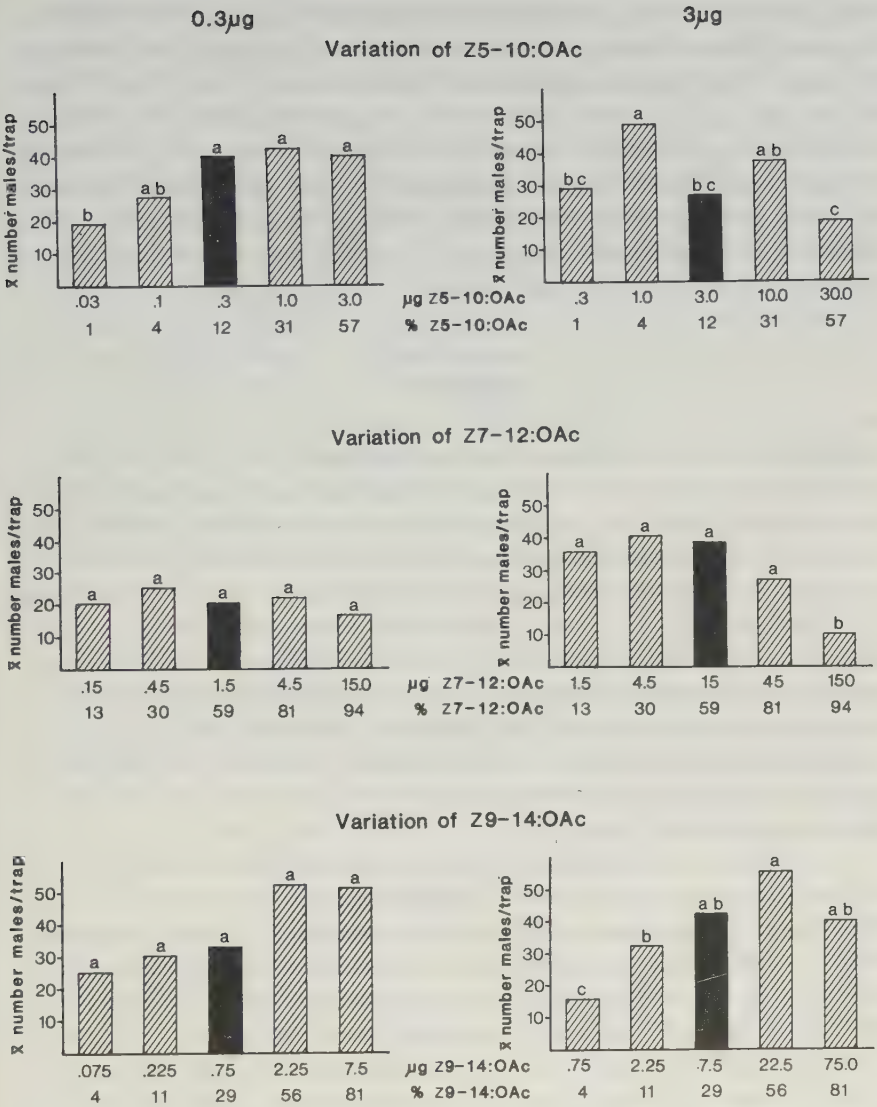

Figure 5. Mean trap catches (n = 5) of male A. segetum in two series of field experiments. In each series the amount of one pheromone component was varied over two orders of magnitude, while the remaining two were kept constant. A 1:5:2.5 mixture of Z5-10:OAc, Z7-12:OAc and Z9-14:OAc was included in each series (=black bar) as a standard. The two dosages are assigned on their content of Z5-10:OAc in the standard bait. The numbers of males trapped are compared within each series by one-way analysis of variance ($\sqrt{X+0.5}$ -transformation) followed by Duncan's multiple range test. Bars labelled with the same letter indicate means that are not significantly different ($P<0.05$).

experiment more extreme ratios between the homologous acetates were tested. The amount of one of the unsaturated compounds was varied over two orders of magnitude, while the remaining two were kept at constant level (Fig. 5). At the low dose (0.3 µg) there was a tendency for highest catches with the highest amounts of Z5-10:OAc and Z9-14:OAc, while the amount of Z7-12:OAc did not seem to be critical at all. At the higher dose (3 µg) highest trap catches were achieved with intermediate amounts of all of the compounds. The difference between the most and the least attractive baits was not more than four times.

DISCUSSION

This study shows a considerable variation between individual female turnip moths in the proportions of the pheromone components. The high coefficients of variation (27 - 83 % after arcsin transformation) indicates a pheromone system very different from the redbanded leafroller, for which Miller & Roelofs (1981) found a precisely regulated isomeric ratio with a 9.7 % cv. Even though Z7-12:OAc generally was the most abundant pheromone component there were actually several females where Z5-10:OAc or Z9-14:OAc was the major constituent (Fig. 3). Specimens were also found in which one or both of these compounds could not be detected. This is remarkable as both field and flight-tunnel experiments have shown a strong decrease in attractivity when one of the components in the three-component mixture is missing (Arn et al. 1983, Löfstedt et al. 1984).

The variation in 10:OAc titre in the wild insects is very striking. Initially we focused on 10:OAc as a gland constituent with possible behavioural activity (Löfstedt et al. 1982). However, parallel to the present investigation of individual variation, detailed behavioural experiments were performed and they did not reveal any activity of this compound (Löfstedt et al. 1984). Further investigations are needed to reveal the true nature of the large peak with retention-time corresponding to that of 10:OAc and the possible behavioural significance of this component.

Shorey and Gaston (1965) found a correlation between pupal weight and subsequent pheromone production, but neither we nor Miller & Roelofs (1981) were able to establish such a correlation between the weight of the imagines and the pheromone titre. Compared to the wild strain-insects the body weight of our laboratory insects was significantly lower but the only significant decrease in a pheromone component in the laboratory strain was observed for Z5-10:OAc. The tendency of a lower pheromone titre in the laboratory insects agrees with what Miller & Roelofs (1981) found in the redbanded leafroller.

Lower pheromone production in laboratory cultures could of course be explained as a result of selection in this direction under laboratory conditions, but a mere loss of vigour due to inbreeding and feeding on synthetic diets is an alternative explanation.

We observed a difference in attractivity between our synthetic blends, approximately covering the range of female variation in pheromone production. Theoretically there should be low additive genetic variance in characters (such as sexual attractants) closely related to fitness (Maynard Smith 1978). A solution to the paradox could be that the female variation documented by us is not genetically determined, contrary to the variation in the redbanded leafroller (Wendell Roelofs, personal communication) and in the European corn borer (Klun & Maini 1979). The pheromone composition could vary with, for instance, female age or time of day. An alternative explanation would be that the range of variation in attractivity observed in this study is evolutionarily unimportant; males can mate several times and all females that produce at least a minimum amount of the pheromone components will eventually become mated. The ultimate test of these hypotheses has to wait until we know the range of ratios actually released by calling females, and these can be precisely reproduced with synthetic dispensers.

We assume that pheromones produced by Lepidoptera in intersegmental glands are given off by passive diffusion. Blend components with different chain length (of different functional moieties) would then evaporate at slightly different rates from the gland surface at different environmental temperatures. Hence the composition of the effluvium from a calling female would vary with temperature. Cardé and Baker (1984) have suggested that this is the raison d'être of several divergent blends in moths, precluding the use of such compounds in very precise ratios. However, this is not true in the case of the turnip moth, although this species uses pheromone components of three different chain-lengths. By use of Raoult's law and vapour pressures extrapolated from Olsson et al. (1983) we calculated the composition of the vapour phase corresponding to a certain female pheromone (gland surface) composition (Table 5). It is obvious that even extreme temperature changes will only cause small changes in the vapour phase composition. For example a temperature shift from 5°C to 20°C or from 20°C to 30°C will, at most, shift the pheromone composition 3 %. This is far less than the range of female produced ratios and the range of blends attractive to males, actually observed in this study. We think that there is an evolutionary cost associated with the production of precise pheromone blends. A precisely regulated pheromone will evolve only when there is a strong selection in favour of species specificity achieved by this means. In the turnip moth the three homologous unsaturated acetates might give enough specificity on a qualitative basis and a regulation of ratios is therefore not critical.

Table 5. Composition of the vapour phase[a] over a liquid mixture of A. segetum pheromone components at different temperatures

	Weight %			
	In liquid phase	In vapour phase		
		5° C	30° C	
Z5-10:OAc	11.7	64.6	61.6	59.7
Z7-12:OAc	58.8	33.6	35.8	37.2
Z9-14:OAc	29.4	1.8	2.5	3.1

a) Calculated from the composition of the liquid phase using Raoult's law and extrapolated P-values from Olsson et al. (1983).

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[1])

PHEROMONE DIALECTS

IN EUROPEAN TURNIP MOTHS *Agrotis segetum*[1)]

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ABSTRACT

Female pheromone gland extracts from cultures of Agrotis segetum, originating from Sweden, France, Hungary and England were analysed for pheromone components and precursors (fatty acids). The pheromone blends were similar in the moths from the Swedish, English and Hungarian populations, whereas the French diverged with a much higher amount of (Z)-5-decenyl acetate relative to the homologous pheromone components (Z)-7-dodecenyl acetate and (Z)-9-tetradecenyl acetate. The frequency of receptor cells sensitive to (Z)-5-decenyl acetate on male antennae, was also highest in the French insects. This is in correspondence with the earlier reported behavioural significance of (Z)-5--decenyl acetate in the French turnip moth, indicating a French pheromone dialect. The biosynthetic basis for the shift in pheromone production as well as ecological and evolutionary implications of the findings are discussed.

INTRODUCTION

The pheromone communication of the turnip moth, Agrotis segetum (Schiff.) (Lep.,Noctuidae) has been extensively studied by several research groups in several countries (Bestmann 1978, Arn et al. 1980 and 1983, Tóth et al. 1980, Löfstedt et al. 1982, Wakamura 1978 and 1980). To try to resolve apparent differences in the identification of the pheromone, Arn et al. (1983) performed an extensive field screening test of different combinations and different ratios of the homologous pheromone components (Z)-5-decenyl acetate (Z5-10:OAc), (Z)-7-dodecenyl acetate (Z7-12:OAc) and (Z)-9-tetradecenyl acetate (Z9-14:OAc). They found that in Denmark, France, Hungary and Switzerland a wide range of three-component mixtures were attractive to male A.segetum. However, the experiments also indicated geographical differences in the response of males, e.g. French A.segetum males were the only ones attracted by Z5-10:OAc alone. Thus the possible existence of A.segetum strains using different pheromones could not be excluded. Bues and Poitout (1981) had already pointed out differences in larval and pupal development between A.segetum populations from various geographical regions.

The homologous pheromone components Z5-10:OAc, Z7-12:OAc and Z9-14:OAc should all be chain-shortening products of (Z)-11-hexadecenoate according to the present concept of pheromone biosynthesis in noctuids (Bjostad & Roelofs 1983). Quantitative differences in pheromone production between turnip moth populations could be reflected in the relative abundances of chain-shortening products in the pheromone gland. We also found it interesting to try to correlate any geographical differences in pheromone production by females and male behavioural responses in the field with relative abundances of pheromone receptors. Löfstedt et al. (1982) reported different male antennal receptor cells specialized to each of the homologous acetates.

In the present study we wanted to determine if there are pheromone differences in European turnip moths by analysis of volatile pheromone gland con-

stituents and pheromone precursors in turnip moth populations from Sweden, France, England and Hungary. We also analysed the frequency of different pheromone receptor cells on the male antennae in the Swedish, English and French populations. A correspondance between male pheromone response and female pheromone production will support the possible existence of European A.segetum pheromone dialects.

MATERIALS & METHODS

Insect material: Insects for the preparation of gland extracts and for electrophysiological recordings were obtained from laboratory cultures established from light-trapped insects. Swedish turnip moths originated from the province of Skåne, southern Sweden. The French culture was obtained from Avignon, Provence, France (courtesy of Dr. R. Bues). English insects originated from Harpenden, Herts, England (courtesy of Dr. P. Sherlock). The Hungarian culture was from the Budapest area (courtesy of Dr. Miklós Tóth). All insects were maintained as described by Löfstedt et al. (1982) on a reversed 16:8 h light:dark cycle.

Preparation of extracts. The pheromone gland (Otto et al. 1976) of 1-4 day old female moths was removed 5-6 h into the scotophase with forceps and placed in a vial with 15 µl solvent. Hexane (redistilled) was used for extraction of volatile gland constituents, whereas total lipid extraction was carried out with 2:1 chloroform: methanol (V/V) (Folch et al. 1957). Prior to use the vials were rinsed with the solvent. Vials for total lipid extraction were treated with NaOH in methanol. The preparations were allowed to extract for at least 20 min before analysis, and extracts were stored in the dark at -20°C. From each population 5-6 extracts in hexane and 5-6 extracts for base methanolysis were prepared. Each extract contained 10 pheromone glands.

Base methanolysis. Base methanolysis was performed to convert fatty acyl moieties to the corresponding methyl esters for gas chromatography (GC) analysis (Litchfield 1972). A sample to be methanolysed was placed in a microvial and evaporated to apparent dryness with a stream of nitrogen. A 0.5 M solution of NaOH in methanol (25 µl) was added to the vial and allowed to react for 20 min at room temperature. A 1.0 M solution of HCl in water (25 µl) and hexane (25 µl) were then added and the sample was shaken for 1 min. The methyl esters contained in the hexane layer were then subjected to GC-analysis.

Gas-chromatography. Capillary gas chromatography with flame ionization detection (FID) was performed on a Hewlett Packard 5880 gas chromatograph equipped with a 30 m x 0.25 mm i.d. Supelcowax 10 fused silica column and on a Hewlett Packard 5830 gas chromatograph equipped with a 42 m x 0.25 mm i.d. fused silica column coated with crosslinked 50 % cyano/25% tolyl siloxan (Mar-

kides et al. 1983). The injector temperature was 250°C. Samples were injected splitless and the split valve was opened 1 min after injection. The column temperature was maintained at 80°C for 2 min after injection and then programmed to 210°C (50% cyano) or 230°C (Supelcowax 10) at 10°C/min. Hydrogen carrier gas was supplied at 40 cm/s (80°C). Sample aliquots (2-8 Female equivalents) were injected on the Supelcowax 10 column for quantification and structual assignments (based on relative retention times; Equivalent Chain Lengths (ECL)) were confirmed by analysis of at least one extract from each population on the more polar 50 % cyano column.

Electrophysiology. Electrophysiological recordings were performed on excised male antennae. EAGs were recorded essentially as described by Van Der Pers (1981). Single-sensillum recordings were done with the tip recording technique as applied by Van Der Pers and Den Otter (1978). The odour sources were disposable syringes containing a piece of filter paper (13 x 13 mm) onto which 1 µg of the test substance had been pipetted in a 100 µl hexane solution. Each randomly selected sensillum trichodeum was stimulated with Z5-10:OAc, Z7-12:OAc and Z9-14:OAc. The receptor cells specialized to these compounds are located in different sensilla (Löfstedt et al. 1982, Van Der Pers & Löfstedt, in press). Thus each sensillum was assigned a specific type according to its response.

Synthetic compounds: Acetate and alcohol standards were available from our laboratory collection of compounds. The alcohols were converted to the corresponding carboxylic acids by adding 1 mg of the alcohol to a solution of pyridinium dichromate (100 mg) in dimethylformamide (1 ml) (Corey and Schmidt 1979) in a 5 ml vial with a teflon-lined screw cap. The mixture was allowed to react at room temperature over night. Diethyl ether (1 ml) and water (3 ml) were added. The organic layer was collected and washed with water. The organic phase was then evaporated to dryness with a stream of nitrogen and the methyl esters of the remaining acid was made by acid methanolysis (Fieser & Fieser 1967). A 1.0 M solution of HCl in methanol (1 ml) was added. The vial was sealed with a teflon-lined screw cap and the mixture was allowed to react at 80°C for 1 h. The methanol was extracted with hexane (2 ml) and the hexane layer was washed with water (1 ml). The hexane phase contained the pure methylesters.

Names of compounds are abbreviated as Z5-10:OAc = (Z)-5-decenyl acetate,

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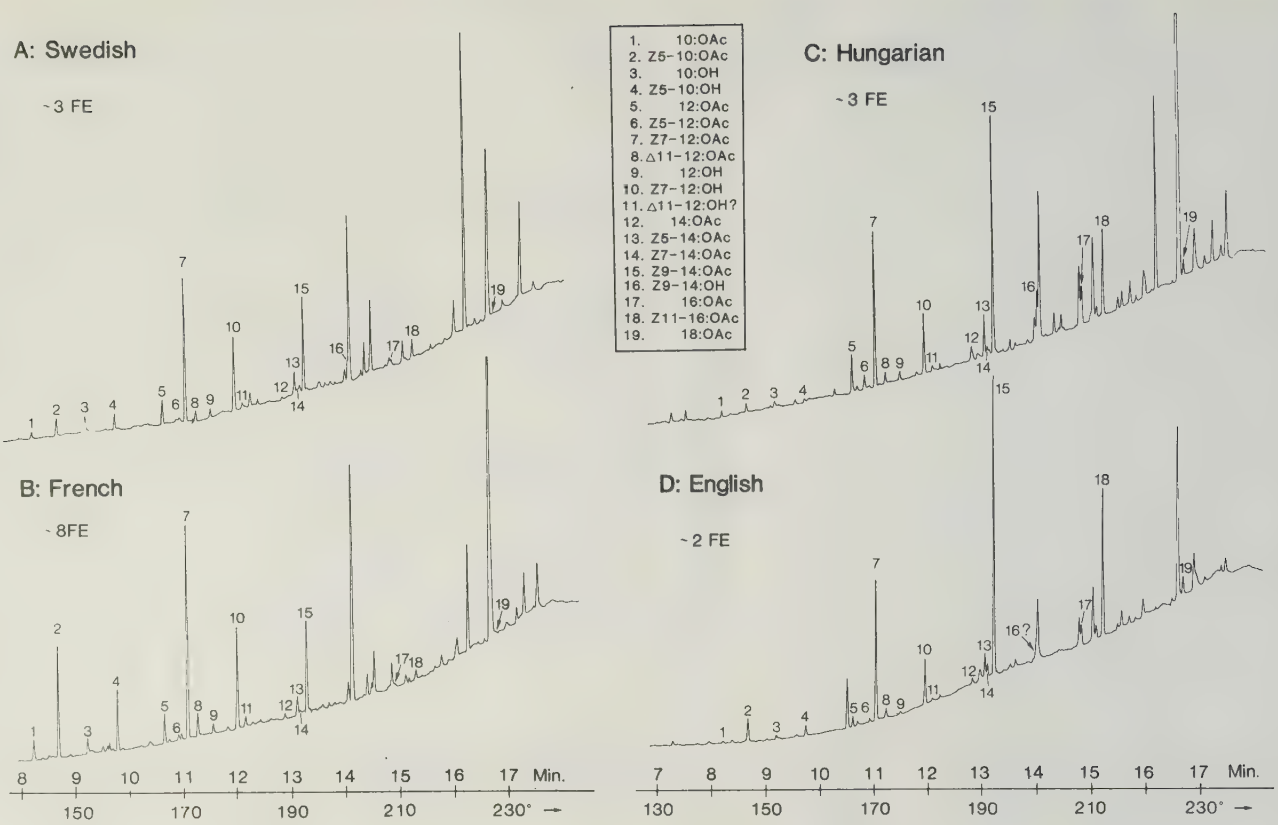
Figure 1. GC-FID chromatogram of female pheromone gland extracts from four European populations of Agrotis segetum, analysed on a 25 m x 0.2 mm i.d. Fused silica column coated with supelcowax 10. (A) Swedish origin, 3 female equivalents (FE), (B) French 8 FE, (C) Hungarian 3FE and (D) English 2FE. Names of compounds are abbreviated. Z5-10:OAc = (Z)-5-decenyl acetate, Z5-10:OH = (Z)-5-decenol, etc.


A: Swedish
~3 FE
B: French
~8FE
C: Hungarian
~3 FE
D: English
~2 FE
1. 10:OAc
2. Z5-10:OAc
3. 10:OH
4. Z5-10:OH
5. 12:OAc
6. Z5-12:OAc
7. Z7-12:OAc
8. Δ11-12:OAc
9. 12:OH
10. Z7-12:OH
11. Δ11-12:OH?
12. 14:OAc
13. Z5-14:OAc
14. Z7-14:OAc
15. Z9-14:OAc
16. Z9-14:OH
17. 16:OAc
18. Z11-16:OAc
19. 18:OAc
16?
8 9 10 11 12 13 14 15 16 17 Min.
150 170 190 210 230° →
7 8 9 10 11 12 13 14 15 16 17 Min.
130 150 170 190 210 230° →

Z5-10:OH = (Z)-5-decenol, Z5-10:Me = (Z)-5-decenoic acid methyl ester, etc.

RESULTS

Acetates and alcohols in pheromone glands. Turnip moth populations from Sweden, France, England and Hungary all contained the same pheromone gland acetates and alcohols (Fig. 1). However, there were significant differences in the relative quantitative composition between the populations (Table 1). The identification of 10:OAc, Z5-10:OAc, Z5-10:OH, 12:OAc, Z7-12:OAc, 11-12:OAc, Z7-12:OH, 14:OAc, Z5-14:OAc, Z9-14:OAc, 16:OAc and Z11-16:OAc from A.segetum ovipositor extracts has already been reported elsewhere (c.f. Löfstedt et al.

Table 1 Relative amounts of straight-chain acetates and alcohols in pheromone gland extracts from four European populations of Agrotis segetum (Z)-7-dodecenyl acetate = 100.

Compound	Swedish	French	Hungarian	English
10:OAc	5± 1	12± 2	4± 1	3± 1
Z5-10:OAc 1)	8± 2 b	116±39 a	6± 1 b	19± 5 b
10:OH	5± 1	26± 8	8± 2	6± 1
Z5-10:OH 1)	6± 2 b	112±32 a	6± 1 b	14± 3 b
12:OAc	26± 5	9± 2	32± 7	11± 2
Z5-12:OAc	2± 0	4± 1	1± 0	2± 1
Z7-12:OAc	100	100	100	100
11-12:OAc	8± 1	8± 2	5± 1	6± 1
12:OH	7± 2	7± 1	17± 4	7± 2
Z7-12:OH 1)	61±14 b	127±26 a	67± 7 b	53±11 b
14:OAc	5± 1	11± 4	15± 3	4± 1
Z5-14:OAc	33±10	11± 2	25± 5	17± 5
Z7-14:OAc	4± 1	8± 3	4± 1	3± 1
Z9-14:OAc 1)	85±11 b	32± 5 c	139±13 a	103±22 ab
16:OAc	14± 3	2±	22± 5	7± 1
Z11-16:OAc 1)	23± 8 a	4± 1 b	37± 6 a	29± 7 a
18:OAc	5± 2	4± 2	7± 3	2± 1

1) Figures in horisontal rows, followed by the same letter are not significantly different by analysis of variance followed by Duncans Multiple Range Test ($P<0.05$).

1982, Lanne et al. 1984). Z5-12:OAc, Z7-14:OAc, 18:OAc, 10:OH and 12:OH were identified by the correspondence between the retention times of synthetic compounds and insect derived material on both the polyethyleneglycol stationary phase and the more polar 50 % cyano/25 % tolyl. Z9-14:OH was not quantified as it could not be resolved from a closely eluting peak (16:Me).

The French population contained more Z5-10:OAc relative to the homologous Z7-12:OAc than the other populations did. This was correlated with a lower relative Z9-14:OAc titre. The Hungarian population showed an opposite tendency, with a low Z5-10:OAc but a high Z9-14:OAc titre. The French population also had a higher titre of Z5-10:OH and Z7-12:OH than the other populations. The remaining compounds with unknown behavioural relevance were not analysed statistically. If the pheromone composition is expressed as percentages of the homologous Z5-10:OAc, Z7-12:OAc and Z9-14:OAc the population differences become even more apparent (Table 2).

Table 2 Female pheromone composition (%) in four European populations of A.segetum[1)]

	Swedish	French	English	Hungarian
Z5-10:OAc	4	47	9	2
Z7-12:OAc	52	40	45	41
Z9-14:OAc	44	13	46	57

1) Gland titre, calculations based on Table 1.

Fatty acyl moities in pheromone glands. Methanolysed gland extracts of the turnip moth populations contained three homologous series of monounsaturated fatty acid methyl esters of probable relevance to the biosynthesis of pheromone components (Fig. 2). From the ECL values on the two columns (Table 3) one series was assigned the following structures: Z5-10:Me, Z7-12:Me, Z9-14:Me and Z11-16:Me. In the same way two other series, i.e. Z5-14:Me, Z7-16: Me and Z9-18:Me as well as Z5-12:Me, Z7-14:Me, Z9-16:Me and Z11-18:Me were assigned. All of the compounds occurred in all of the populations, and only minor quantitative differences were revealed (Table 4). In the series Z5-10:Me, Z7-12:Me, Z9-14:Me and Z11-16:Me related to the behaviorally active homologous acetates Z5-10:OAc, Z7-12:OAc and Z9-14:OAc, there were no statistically significant differences.

Responses of male antennae to pheromone components. In the first experiment, single sensillum responses were recorded from 25 French and 25 English

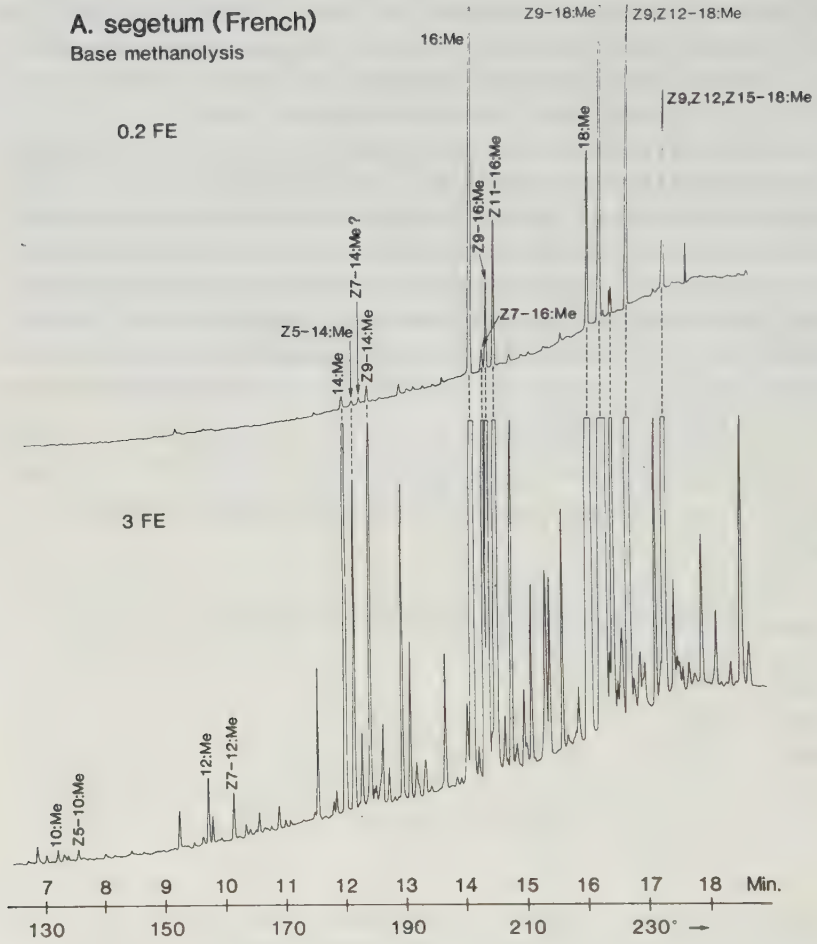


Figure 2: GC-FID chromatogram of a French Agrotis segetum gland extract after base methanolysis, analysed on a 25 m x 0.2 mm i.d. fused silica coated column with Supelcowax 10. Names of compounds are abbreviated. Z5-10:Me = methyl (Z)-5-decenoate, etc, FE = female equivalents.

sensilla, for comparison with an earlier report on the sensory equipment of Swedish males. The highest number of Z5-10:OAc cells was found in the French population, but the 95% confidence intervals for the Swedish and the French populations overlapped (Table 5).

To substantiate the difference between the Swedish and the French populations, a second experiment was performed. Again we found a higher proportion

Table 3 Equivalent chain length[1)] of pheromone precursor candidates in A.segetum and synthetic methyl esters on GC-columns of different polarity

	Supelcowax 10		50% Cyano/25 % tolyl	
	A.segetum	Reference	A.segetum	Reference
10:Me	1000	1000	1000	1000
Z5-10:Me	1027	1027	1027	1027
12:Me	1200	1200	1200	1200
Z5-12:Me	1219	1218	1220	1220
Z7-12:Me	1237	1237	1242	1243
11-12:Me	1256	1255	-	1256
14:Me	1400	1400	1400	1400
Z5-14:Me	1415	1415	1417	1417
Z7-14:Me	1427	1427	1432	1434
Z9-14:Me	1439	1439	1447	1448
16:Me	1600	1600	1600	1600
Z7-16:Me	1619	1621	1628	1629
Z9-16:Me	1625	1628	1636	1639
Z11-16:Me	1639	1640	1649	1650
18:Me	1800	1800	1800	1800
Z9-18:Me	1819	1820	1828	1828

1) Relative to straight-chain saturated methyl esters

of Z5-10:OAc receptor cells in the French population compared to the Swedish, 87 vs 66%. Due to the higher number of recordings this time the difference became statistically significant (Table 5). EAG-recordings over four orders of magnitude (10^{-3} to 10 µg) did not reveal any corresponding differences in the EAG-responses. The EAG amplitudes were identical for both populations, testing all three pheromone components.

DISCUSSION

This study confirms the existence of pheromone dialects in European populations of the turnip moth A.segetum. The aberrant male response of French turnip moths to pure Z5-10:OAc reported by Arn et al. (1983) is correlated with our finding of a higher frequency of Z5-10:OAc receptors on the male antennae and a higher relative titre of this component in the French females. Furthermore our analysis of the postulated pheromone precursors indicates a

Table 4 Relative amounts of methylesters in pheromone gland extracts from four European populations of Agrotis segetum. Z11-16:Me = 100

Compound	Swedish	French	Hungarian	English
	n=6	n=5	n=5	n=5
10:Me	0.6± 0.2	0.4± 0.1	0.2± 0.1	0.8± 0.5
Z5-10:Me 1)	0.2± 0.0 a	0.2± 0.0 ab	0.1± 0.1 ab	0.1± 0.0 b
12:Me	1.7± 0.4	2.6± 0.7	1.4± 0.2	3.2± 1.2
Z7-12:Me 1)	1.2± 0.3 a	0.8± 0.1 a	1.0± 0.2 a	0.8± 0.2 a
14:Me	26.0± 6.0	23.0± 4.9	13.2± 2	31.2± 7.7
Z5-14:Me	9.3± 1.5	7.6± 1.0	6.4± 0.9	8.7± 1.7
Z7-14:Me	1.0± 0.1	1.9± 0.3	1.3± 0.3	2.0± 0.8
Z9-14:Me 1)	18.8± 2.8 a	11.6± 1.2 a	15.1± 2,7 a	17.1± 1.8 a
16:Me	577 ±82	1152 ±269	512 ± 39	1468 ±458
Z7-16:Me	14.6± 1.0	19.9± 2.6	14.3± 0.8	28.0± 5.4
Z9-16:Me	78.4±12.7	161 ± 42	69.3± 6.1	192 ± 57.5
Z11-16:Me	100	100	100	100
18:Me	96.4± 4.1	129 ± 15	13.3±18	192 ± 31
Z9-18:Me	601 ± 58	1029 ±127	717 ±92	1379 ±338
Z11-18:Me	6.1± 0.5	9.2± 2.7	6.6± 1	13.0± 3.1

1) Figures in horisontal rows, followed by the same letter are not significantly different by analysis of variance followed by Duncan's Multiple Range Test($P<0.05$)

possible biosynthetic mechanism for the observed difference in female pheromone production. The significant difference in female pheromone production between the French and the other populations, is not correlated with a clear shift in the relative titre of chain-shortening products, fatty acids postulated as pheromone precursors. Thus it seems to us that the biosynthetic basis for the population differences could be a differential reduction of the acids to alcohols. Also with respect to the probable subsequent and final biosynthetic step, the acetylation of the alcohol, the French insects seem to diverge from the rest. The titre of Z5-10:OH ad Z7-12:OH is higher in the French females than in the others. The possible behavioural significance of this finding was not the subject of the study.

Geographical differences in responses to pheromones have earlier been reported in some insects. Populations of the European corn borer Ostrinia nubilalis, introduced to North America in the early 1900s, vary in their production of and response to the two pheromone components Z11-14:OAc and

E11-14:OAc (Klun et al. 1973, Kochansky et al. 1975). The so-called Z- and E-strains produce and are preferentially attracted to 97:3 and 4:96 ratios of the two acetates, respectively. The EAG responses of the two strains and hybrids thereof were investigated by Nagai et al. (1977). Whereas the Z11-14:OAc elicited a significantly higher response than the E-isomer from the Z-strain, responses to both these isomers were similar with the antennae from the E-strain and the hybrids. The shift in relative EAG sensitivity could be due to a shift in the frequency of cells specialized to the respective isomers, analogous to the shift in receptor frequencies that we found in the turnip moth.

In the North American bark beetle Ips pini, eastern and western populations prefer the pheromone of their own populations (Lanier et al. 1972). Western I. pini produce and respond to (-) ipsdienol as their pheromone, whereas eastern beetles produce a 65:35 ratio of (+):(-) ipsdienol and respond more strongly to the racemate in the field (Lanier et al. 1980). Mustaparta et al. (1980) attempted to correlate these differences with the number of (+) and (-) ipsdienol cells on the antennae. In the western population 9 cells were specialized to the (-) ipsdienol enantiomer and one to the (+) whereas in the eastern population the number of cells were 6 and 3, respectively. However, the low number of cells tested, did not allow any statistically significant discrimination between beetles from the populations.

Table 5 Frequencies of pheromone receptor cells in sensilla trichodea on antennae of male A.segetum from different populations

	% (95% confidence interval)		
	Z5-10:OAc	Z7-12:OAc	Z9-14:OAc
1st experiment:			
Swedish (n=98) 1)	69(59-77)	28(20-37)	2(0.5- 7)
French (n=25)	84(65-94)	16(6-34)	0(0 -13)
English (n=25)	40(23-59)	56(37-73)	4(0.7-20)
2nd experiment:			
Swedish (n=99) 2)	66(56-75)	33(25-43)	1(0 - 5)
French (n=99) 2)	87(79-92)	12(7-19)	1(0 - 5)

1) Data from Löfstedt et al. (1982). In this experiment two additional cells, which did not respond to any of the tested compounds, were recorded from.

2) In this experiment one additional cell was recorded from, which did not respond to any of the tested compounds.

The correlation between pheromone production, pheromone perception and behavioural response in A.segetum, as well as O.nubilalis and I.pini, is interesting. Alexander (1962) and O'Donald (1962) suggested from a theoretical point of view that species-specific features of communication systems should be inherited as blocks of co-adapted gene complexes or supergenes. Evidence for such a mechanism was obtained when Grula & Taylor (1979) analysed the inheritance of 13-methyl heptacosane production in a sulphur butterfly Colias eurytheme. The X-chromosome carried most of the genes (or the gene) for the production of this pheromone component, as well as visual components of the communication system. Whether such a gene complex accounts for the pattern observed in the turnip moth remains to be investigated. Gas chromatographic analysis of the pheromone composition in individual females and quantification of the male phenotype by single sensillum recordings, as described in this paper, should provide means for an approach to this problem by crossing experiments.

Pheromone dialects in the turnip moth could be the outcome of non-adaptive random shifts in the pheromone communication system or alternatively the result of adaptation to different interspecific interactions within the species range of distribution. Examples of character displacement or character release would lend support to the adaptive explanation. For example the leaf-roller moth Archips argyrospilus in New York is more acutely tuned to blend proportions than the population in British Columbia, where the sibling competitor A.mortuanus is missing (Cardé et al. 1977). Using the same compounds in their pheromones, these species compete for the same communication niche. In the case of the turnip moth Arn et al. (1983) did indeed observe the attraction of other noctuid species to combinations of Z5-10:OAc, Z7-12:OAc and Z9-14:OAc, in the four European localities investigated by them. Their data, however, does not allow any discrimination between the French locality and the others in this respect. As pheromone differences between the European moth communities might be subtle and include additional components, testing of the adaptation hypothesis will not be easy. This important experiment on the role of adaptation in pheromone variation is probably more easily accomplished by a comparison between European and Japanese A.segetum. The difference in pheromones between European A.segetum and the Japanese A.fucosa, recently synonymized with A.segetum by taxonomists (S. Wakamura, personal communication), is not only quantitative but qualitative. The Japanese pheromone is a mixture between Z5-10:OAc and Z7-10:OAc (Wakamura 1978, 1980). This isomer blend and the European homologous three-component blend should be tested both in Japan and in Europe. Attraction of other species than A.segetum may give an adaptive explanation for the geographical variation.

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Tryckericentralen

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